AN ABSTRACT OF THE DISSERTATION OF

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Abstract approved: _____
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Oceanic crust covers nearly 70% of the Earth's surface, of which, the upper, sediment layer is estimated to harbor substantial microbial biomass. Marine crust; however, extends several kilometers beyond this surficial layer, and includes the basalt and gabbro layers. In particular, the basalt layer has high permeabilities which allows for infiltration and circulation of large volumes of seawater. Seawater interacts with the host rocks and can result in abiotic hydrogen, methane, and other low molecular weight carbon compounds. Endoliths residing in this environment are; therefore, uniquely poised to take advantage of the by-products of this reaction.

Whether the resident prokaryotic communities in lithic crust utilize abiotically produced volatiles, such as methane, is unknown. Further, little is known about the global distribution of basalt endoliths. To date, gabbroic microflora have not yet been examined. The gabbroic layer may; therefore, harbor great microbial and metabolic diversity. To this end molecular and bioinformatics techniques were used to examine the microbial communities associated with basalt and gabbro.

Cloning and sequencing of 16S rDNA from basalt and gabbro samples revealed that a disparate microbial communities resides in these two environments.

Basalt samples harbor a surprising diversity of seemingly cosmopolitan microorganisms, some of which appear to be basalt specialists. Conversely, gabbros have a low diversity of endoliths, none of which appear to be specifically adapted to the gabbroic environment.

Despite the differences in the microbial communities in basalt and gabbro, analysis of functional genes using a microarray revealed overlapping metabolic processes. Genes coding for carbon fixation, methane generation and oxidation, nitrogen fixation, and denitrification were present in both rock types. None of these metabolic processes have been reported previously in basalt or gabbro hosted environments. Taken together, these findings provide significant insight into the possible biogeochemical cycling occurring in marine crust.

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by
Olivia Underwood Mason

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Olivia Underwood Mason, Author

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CONTRIBUTION OF AUTHORS

For Chapter 2 Ulrich Stingl provided advice and assistance with the phylogenetic analyses. Larry J. Wilhelm carried out automated BLAST searches on 16S rDNA sequences from basalts to determine nearest neighbors. Markus M. Moeseneder established basalt enrichment cultures and did DNA extractions for the cultures that were analyzed in this chapter. Carol A. Di Meo-Savoie assisted with basalt sample collection. Martin R. Fisk and Stephen J. Giovannoni helped design experiments, assisted with data analysis, and manuscript preparation.

For Chapter 3 Carol A. Di Meo-Savoie collected samples, extracted DNA, and did the T-RFLP and UPGMA analyses. Joy D. Van Nostrand and Jizhong Zhou assisted with the microarray analysis of functional genes. Martin R. Fisk and Stephen J. Giovannoni helped design experiments, assisted with data analysis, and manuscript preparation.

For Chapter 4 Tatsunori Nakagawa collected microbiological samples during Expedition 304 and did the molecular analysis of these samples with the assistance of Akihiko Maruyama. Martin Rosner participated in Expedition 305 and carried out the geochemical analysis. Joy D. Van Nostrand and Jizhong Zhou assisted with the microarray analysis. Martin R. Fisk and Stephen J. Giovannoni helped design experiments, assisted with data analysis, and manuscript preparation.

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Prokaryotes Associated with Marine Crust

CHAPTER 1
DISSERTATION INTRODUCTION

Ocean crust covers nearly 70% of the Earth's surface and is composed of a series of layers: sediment, basalt and gabbro (Figure 1.1), through which the entire ocean is circulated once every million years (Edwards et al., 2003a). In the lithic portion of marine crust basalts have high permeabilities enabling infiltration and circulation of large quantities of seawater (Fisher, 1998; Fisher and Becker, 2000). The abiotic reaction between seawater and basalt crust results in a significant flux of energy and solutes to the overlying seawater (Fisher, 1998). Thus fluid rock interactions and the resulting alteration processes have a significant influence on ocean chemistry.

The role that microorganisms play in alteration processes has been the focus of numerous studies, with compelling evidence suggesting that they do play a part in alteration (Thorseth et al., 1995; Giovannoni et al., 1996; Fisk et al., 1998; Torsvik et al., 1998; Furnes and Staudigel, 1999; Furnes et al., 2001; Banerjee and Muehlenbachs, 2003; Fisk et al., 2003; Furnes et al., 2004). To date; however, their role in biogeochemical cycling remains largely unknown. Further, the microflora in gabbroic rocks have not yet been analyzed; therefore, this layer could harbor previously unrecognized microbial and metabolic diversity.

Recent quantitative analyses revealed that basalts harbor 6×10^5 to 4×10^6 and 3×10^6 to 1×10^9 cells g-1 (Einen et al., 2008; Santelli et al., 2008). Given the substantial volume of habitable volcanic ocean crust ($\sim 7 \times 10^{17}$ m^3) these cell densities

Figure 1.1. Stylized cross section of oceanic crust.

This figure was modified from Kennett, J.P. (1982) *Marine Geology*. Englewood Cliffs, NJ: Prentice-Hall, figure 7-4, pg 211.

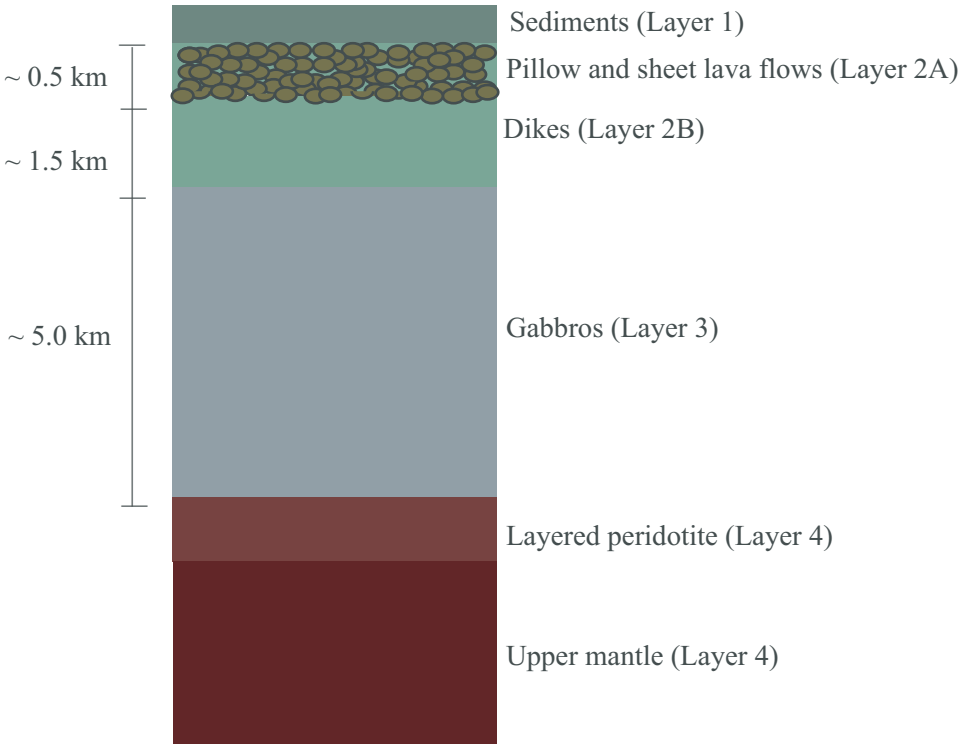
Sediments (Layer 1)
Pillow and sheet lava flows (Layer 2A)
Dikes (Layer 2B)
Gabbros (Layer 3)
Layered peridotite (Layer 4)
Upper mantle (Layer 4)
~ 0.5 km
~ 1.5 km
~ 5.0 km

Figure 1.1.

are significant. Santelli et al. (2008) reported that prokaryotic cell abundances are 3-4 orders of magnitude greater in basalt than in the overlying seawater. Einen et al. (2008) suggested that the total number of microorganisms present in ocean crust exceeds the number present in seawater. Given these cellular abundances in conjunction with the massive volume of habitable ocean crust it is requisite that this environment be elucidated to understand biogeochemical cycling in the deep sea, and how these subsurface processes affect the overlying hydrosphere.

Several studies reported surprising microbial diversity in environmental basalt samples from several different geographic locations (Thorseth et al., 2001; Fisk et al., 2003; Lysnes et al., 2004a; Lysnes et al., 2004b). Fourteen bacterial and archaeal phyla and subphyla were reported in all. These studies examined basalt samples from disparate sites. No consensus had emerged regarding dominance or distribution of the different phylotypes. To identify patterns in the distribution of the different clades a comprehensive phylogenetic analysis of all 16S rDNA sequences from basalt samples was needed.

The majority of phyla associated with basalts are not closely related to characterized microorganisms making it challenging to infer their metabolic activity from phylogenetic relationships. The chemical composition of igneous rocks does provide some clues, as fresh basalts are ~ 8 % wt FeO and 2 % wt Fe_2O_3; therefore, niches for both iron-oxidizers and reducers are present. In fact, Edwards et al. (2003b) isolated Fe-oxidizing bacteria that are capable of growth on basalt glass from metalliferous sediments and sulfides. Basalts also contain ~ 0.2 % wt MnO. Templeton et al. (2005) successfully isolated manganese-oxidizing bacteria from Loihi

Seamount basalts. These cultured representatives suggest that Fe- and Mn-oxidation is occurring in marine basalts. Yet, the majority of microorganisms are not yet cultured (Rappe and Giovannoni, 2003), including basalt endoliths; therefore, the diversity of metabolic processes in basalt are largely unknown. To circumvent the lack of cultured representatives from basalt, a molecular approach is needed to evaluate the functional genes present in this environment. This analysis would provide a greater understanding of the potential biogeochemical cycling in this environment.

The diversity, distribution, and function of the microorganisms associated with the gabbroic layer of ocean crust have not yet been reported. Volumetrically, this layer represents the majority of ocean crust (~60%), yet much of this is inhabitable, reaching temperatures that exceed the current known maximum temperature of 121 °C at which microorganisms can survive (Kashefi and Lovley, 2003). However, in places the gabbroic layer is exposed on the seafloor allowing for colonization of a large volume of this habitat. The chemical similarity between basalts and gabbros suggests that the habitable zone in gabbro, and what is exposed at the seafloor, may harbor a diversity of microorganisms similar to basalt, some of which may be capable of Fe- and Mn-oxidation. To date; however, there is no data on gabbroic endoliths.

Gabbros can be exposed on the seafloor near spreading axes, where detachment faulting leads to the formation an ocean core complex characterized as having a gabbroic central dome (Ildefonse et al., 2007). Examples of ocean core complexes are the Atlantis Bank, at the Southwest Indian Ridge (SWIR) and the Atlantis Massif, at the Mid-Atlantic Ridge (Ildefonse et al., 2007). At the gabbroic

central dome of Atlantis Bank ocean core complex (Hole 735B) geochemical analysis of fluid inclusions revealed abundant abiotic methane, with some samples containing up to 40 mol % methane (Kelley, 1996; Kelley and Früh-Green, 1999). Abiotic methane could provide carbon and energy for growth in this environment. In fact, abiotically produced volatiles, such as methane at the Lost City Hydrothermal Field (30 °N, 42 °W, Atlantis Massif) (Proskurowski et al., 2008), have been shown to sustain prokaryotes (Kelley et al., 2001). Whether microorganisms are sustained by abiotic methane in gabbros remains unknown.

Methods

Terminal restriction fragment length polymorphism

Terminal restriction fragment length polymorphism (T-RFLP) has been used to examine microbial diversity in numerous environments, such as in aquifer sand, sludge, termite guts (Liu et al., 1997), in the marine environment (Moeseneder et al., 1999), and in soil (Osborn et al., 2000). This technique provides an overview, or a fingerprint, of microbial diversity and allows for comparisons of microbial community composition in diverse samples. Moeseneder et al. (1999) used T-RFLP analysis and demonstrated that bacterioplankton differed in the South and North Aegean Sea. Similarly, T-RFLP profiles were generated for basalt and gabbro samples to assess diversity and to evaluate community structure in Chapters 3 and 4.

Multiple Displacement Amplification

Multiple displacement amplification (MDA) utilizes the φ29 DNA polymerase in isothermal amplification of genomic DNA (Lage et al., 2003). The ability to increase genomic DNA from a variety of sample types has many applications. Recently MDA was used to increase the quantity of archaeal and bacterial genomic DNA following fluorescence *in situ* hybridization and immunomagnetic cell capture from methane rich marine sediments (Pernthaler et al., 2008). MDA amplified DNA was then sequenced providing significant insight into methane cycling by uncultured microorganisms. MDA has also been used to study environments with low prokaryotic cell densities, such as in corals (Yokouchi et al., 2006). This method is particularly appropriate for studying hard rock environments that may have low cell densities.

Functional genes

The presence of functional genes indicates the potential metabolic diversity in an environment. However, in an environmental sample assaying for all known functional genes using the polymerase chain reaction, cloning, and sequencing is not realistic. Recently, a DNA based microarray, GeoChip, was constructed that allows for detection of >10,000 genes in >150 functional groups involved in nitrogen, carbon, sulfur, and phosphorus cycling (He et al., 2007). Additionally, genes coding for metal reduction and resistance, and organic contaminant degradation are present on the microarray (He et al., 2007). The functional genes present in MDA amplified genomic DNA are assayed for simultaneously in a single sample. This approach has been used to analyze functional genes in groundwater and in Antarctic soils (Wu et al.,

2006; Yergeau et al., 2007) and is particularly useful in studying hard rock samples, where functional genes have not yet been analyzed.

Cloning, Sequencing and Phylogenetic Reconstruction

Phylogenetic reconstruction using cloning and sequencing of 16S rRNA gene sequences has been used in marine and freshwater environments to examine microbial distribution and dominant phylotypes (Massana et al., 2000; Rappe et al., 2000; Zwart et al., 2002; Takai et al., 2004). Massana et al. (2000) examined the distribution of planktonic Archaea in surface and aphotic zone samples. Phylogenetic reconstruction revealed that some of the Archaea were found in all samples and suggested that these microorganisms were ubiquitous, while other Archaea exhibited differential distributions. For example, the MGI-α sub-clade delineated in this study dominated shallow samples verses the MGI-γ which dominated deeper samples collected from the aphotic zone. Similarly, phylogenetic reconstruction can be used to examine microbial distribution and dominant phylotypes in lithic ocean crust.

Objectives

The purpose of the research carried out for this dissertation was to use multiple approaches to analyze the prokaryotic communities associated with marine crust. In Chapter 2 cloning, sequencing, and phylogenetic reconstruction were used to examine the distribution and dominant phylotypes of microorganisms associated with basalts collected from disparate locations. In Chapter 3, T-RFLP was used to analyze

microbial diversity and to compare basalt and deep sea microflora. Cloning and sequencing was used to determine which bacterial and archaeal species are present in basalts collected from different geographic locations. Microbial distribution was analyzed using phylogenetic reconstruction. Finally, functional genes in MDA amplified genomic DNA were analyzed using the GeoChip microarray. Similar to Chapter 3, T-RFLP, cloning and sequencing, phylogenetic reconstruction, and microarray analyses were used to examine gabbroic endoliths in Chapter 4.

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PHYLOGENY OF ENDOLITHIC MICROBES ASSOCIATED WITH MARINE BASALTS

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Abstract

We examined the phylogenetic diversity of microbial communities associated with marine basalts, using over 300 publicly available 16S rDNA sequences and new sequence data from basalt enrichment cultures. Phylogenetic analysis provided support for 11 monophyletic clades originating from ocean crust (sediment, basalt and gabbro). Seven of the ocean crust clades (OCC) are bacterial, while the remaining four OCC are in the Marine Group I (MGI) Crenarchaeota. Most of the OCC were found at diverse geographic sites, suggesting that these microorganisms have cosmopolitan distributions. One OCC in the Crenarchaeota consisted of sequences derived entirely from basalts. The remaining OCC were found in both basalts and sediments. The MGI Crenarchaeota were observed in all studies where archaeal diversity was evaluated. These results demonstrate that basalts are occupied by cosmopolitan clades of microorganisms that are also found in marine sediments but are distinct from microorganisms found in other marine habitats, and that one OCC in the ubiquitous MGI Crenarchaeota clade may be an ecotype specifically adapted to basalt.

Introduction

Basalts are the most abundant rock near the Earth's surface. At mid-ocean ridges approximately 20 km^3 of basalt is produced on an annual basis (Parsons, 1981). Abiotic basalt alteration results in elemental exchange between basalt and seawater on a vast scale, significantly influencing ocean chemistry. Numerous studies have focused on the role of microorganisms in basalt alteration. Several reported textural, genetic and chemical evidence suggesting a biological role in alteration (Thorseth et al., 1995; Giovannoni et al., 1996; Fisk et al., 1998; 2003; Torsvik et al., 1998; Furnes and Staudigel, 1999; Furnes et al., 2001; 2004; Banerjee and Muehlenbachs, 2003). However, the specific mechanism(s) of biotic basalt alteration and the microorganisms responsible remain elusive.

Recent studies reported microbial diversity in basalt samples from seven different geographic locations (Thorseth et al., 2001; Fisk et al., 2003; Lysnes et al., 2004a,b; C.A. Di Meo-Savoie, M.M. Moeseneder, K.L. Vergin, M.R. Fisk and S.J. Giovannoni, unpublished). In all, 14 bacterial and archaeal phyla and subphyla were observed. Enrichment culture studies in which basalt alteration and dissolution was monitored (Daughney et al., 2004; Einen et al., 2006; M.M. Moeseneder, C.A. Di Meo-Savoie, J. Durnin, J. Josef, O.U. Mason, M.R. Fisk and S. J Giovannoni, unpublished) reported enhanced dissolution in the presence of Gammaproteobacteria (Daughney et al., 2004) and an assemblage of *Bacteria* and *Archaea* (M.M. Moeseneder, C.A. Di Meo-Savoie, J. Durnin, J. Josef, O.U. Mason, M.R. Fisk and S.J. Giovannoni, unpublished). Einen et al. (2006) observed similar alteration morphology in both enrichment cultures and abiotic controls, suggesting that chemistry plays an

important role. Other efforts led to the isolation of Fe-oxidizing bacteria from metalliferous sediments and sulfides (Edwards et al., 2003). These microorganisms are able to grow on basalt glass (Edwards et al., 2003). Additionally, Mn-oxidizing bacteria have been isolated from Loihi Seamount (Templeton et al., 2005). In total, these studies revealed a diverse microflora implicated in basalt dissolution, but no consensus has emerged about the dominant phylotypes found in basalts or their distributions.

Our goals were to apply phylogenetic reconstruction to (i) identify monophyletic clades that are found only in ocean crust environments (sediment, basalt and gabbro), (ii) determine whether these clades are broadly distributed or are endemic to individual basalt outcrops, and (iii) screen basalt enrichment cultures for these clades. To accomplish this we compiled all published 16S rDNA sequences from basalts (Table 2.1) and examined four basalt enrichment cultures (M.M. Moeseneder, C.A. Di Meo-Savoie, J. Durnin, J. Josef, O.U. Mason, M.R. Fisk and S.J. Giovannoni, unpublished) by cloning, restriction fragment length polymorphism (RFLP) screening and sequencing. The analysis revealed a single archaeal clade that was only recovered from basalts, and multiple clades of *Bacteria* and *Archaea* that are found exclusively in ocean crust. Further, we determined that the majority of these clades are cosmopolitan in their distribution. Several of the ocean crust archaeal clades, but none of the ocean crust bacterial clades, were present in the enrichment cultures.

Methods

Sampling and nucleic acid extraction

Table 2.1. Cultivation- and non-cultivation-based studies of the microbial communities associated with marine basalts.

Table 2.1 Cultivation- and non-cultivation-based studies of the microbial communities associated with marine basalts.

Sequence identifier[a]	Collection site	Sample type	Method	16S rDNA sequences analysed	Sequence length	References
DQ0707, DQ0708	East Pacific Rise, 9°N	Environmental	Clone library	36 (B and A)[b]	8–1492 (B) 6–915 (A)	C.A. Di Meo-Savoie, M.M. Moeseneder, K.L. Vergin, M.R. Fisk and S.J. Giovannoni (unpublished)
DQ0707, DQ0708	Cobb Seamount	Environmental	Clone library	27 (B and A)	8–1492 (B) 6–915 (A)	C.A. Di Meo-Savoie et al. (unpublished)
AY2678	Hilo, Hawaii	Environmental	Clone library	12 (A)	6–915 (A)	Fisk et al. (2003)
AF2493	Knipovich Ridge	Environmental	DGGE	12 (B and A)	338–518 (B) 340–519 (A)	Thorseth et al. (2001)
AY4638, AY4639	Kolbeinsey, Mohns, and Knipovich Ridges	Environmental and enrichment cultures	DGGE	76 (B and A)	338–518 (B) 340–519 (A)	Lysnes et al. (2004a)
AY1298, AY1299, AY6829	Southeast Indian Ridge	Environmental and enrichment cultures	DGGE	49 (B)	338–518 (B)	Lysnes et al. (2004b)
DQ3156	Mohns Ridge	Enrichment cultures	DGGE	62 (B)	8–518 (B)	Einen et al. (2006)
EF067 and D3816, BECC1233a[c] and BECC1196b	CoAxial segment of the Juan de Fuca Ridge	Enrichment cultures	Clone library	12 (A)	6–915 (A)	This study
EF067, EF5812 and D3825, BECC1447a[d] and BECC1442a	Brown Bear Seamount	Enrichment cultures	Clone library	19 (B)	8–1492 (B)	This study
DQ4120 and KBB, LOB, SPB	Loihi Seamount	Isolates	Direct sequencing of isolates	22 (B)	8–1492 (B)	Templeton et al. (2005)

a. Prefix is listed. Any combination of numbers or letters follows the prefix to identify individual sequences.
b. (B) Bacteria and (A) Archaea.
c. BECC indicates a basalt enrichment culture clone, 1233a and 1196b are sample names, and a is aerobic, b is anaerobic.
d. 1447a and 1442a are sample names.

Basalts samples were collected at the CoAxial segment of the Juan de Fuca Ridge and from neighbouring seamounts using the Deep Submergence Vehicle (DSV) *Alvin*. Basalt glass was obtained from basalt samples and crushed into 5–40 mm pieces. Enrichment cultures, containing artificial seawater media (see Appendix A), were inoculated with 0.5 g of crushed basalt glass. Enrichment cultures were subsampled and DNA was extracted using an Ultraclean Soil DNA kit (Mo Bio Laboratories, Carlsbad, CA) following the manufacturer's protocol. DNA from four of these enrichment cultures, representing two DSV *Alvin* dives (D3816F, CoAxial segment of the Juan de Fuca Ridge and D3825, Brown Bear Seamount), were analysed here (sequence prefixes D3816F, BECC1233a- and BECC1196b- and D3825, BECC1442a- and BECC1447a-).

PCR, clone library construction, RFLP analysis and sequencing

Bacterial 16S rDNA extracted from two enrichment cultures inoculated with basalts collected during dive D3825 were amplified in (final concentrations) 1× *Taq* Buffer (Fermentas), 0.5 U *Taq* DNA polymerase (Fermentas), 1.5 mM $MgCl_2$, 1 mM dNTP mixture and 250 nM of 27F-B (5'-AGRGTTYGATYMTGGCTCAG-3') and 1492R (Lane, 1991) with 1 ng μl^{-1} template DNA added to each reaction. Amplifications were carried out in a PTC-200 Thermal Cycler (MJ Research, Watertown, MA, USA) with the following conditions: 35 cycles of 94°C for 15 s, 55°C for 1 min and 72°C for 2 min, with a final extension of 72°C for 5 min. Archaeal rDNA from two enrichment cultures inoculated with basalts from dive D3816F were

amplified with the same conditions as above, with the following modifications: (final concentrations) 2.5 mM $MgCl_2$ and 200 nM each of the archaeal-specific primer 20F (Massana et al., 1997) and the universal primer 1492R were used in PCR reactions. The same thermal cycler conditions were used for archaeal amplifications, with 30 cycles instead of 35. Following the initial archaeal amplifications a semi-nested PCR was performed with 20F and the archaeal-specific primer 915R (Stahl and Amann, 1991) using the identical thermal cycler conditions to the initial archaeal PCR reactions. Clone library construction, screening and processing were carried out as previously described (Vergin et al., 2001). Briefly, bacterial and archaeal amplification products were cloned into pGEM-T Easy Vector (Promega) and 16S rDNA inserts were amplified with M13 primers. Full-length inserts were characterized by RFLP analysis. Inserts were digested with the restriction enzyme BsuR1 (HaeIII) (Fermentas) for 3 h at 37°C with the appropriate buffer, 10 mM $MgCl_2$ (final concentration) and 10 units of HaeIII. Digested PCR products were resolved on a 3% agarose gel. One clone from each unique RFLP pattern was sequenced with 519R (5'-GWATTACCGCGGCKGCTG-3') on an ABI 3730 capillary sequencer. Near full-length sequences were generated for clones that were similar to previously published basalt sequences. Chimeric sequences were identified with Pintail (Ashelford et al., 2005) and Mallard (Ashelford et al., 2006).

Nucleotide sequence accession numbers

Archaeal and bacterial 16S rDNA sequences generated for this study were submitted to the GenBank (Benson et al., 2005) database under the Accession No. EF067896–EF067915 and EF581286–EF581296.

Phylogenetic analysis

Ribosomal RNA gene sequences from cultivation- and non-cultivation-based studies of basalts (Table 2.1) were searched against the GenBank (Benson et al., 2005) database to identify sequences with high similarity (lowest expect value and highest bit score) to the queried sequence. All basalt sequences and nearest neighbours were imported into an ARB (Ludwig et al., 2004) database that contains 50 000 near full-length 16S rDNA sequences. Sequences were aligned using the automatic alignment function available in the ARB (Ludwig et al., 2004) sequence analysis software package. Sequence alignments were verified using similar sequences as a reference and manually edited when necessary. All phylogenetic analyses were performed using ARB (Ludwig et al., 2004). Near full-length sequences, consisting of at least 1200 nucleotides for *Bacteria* and at least 800 nucleotides for *Archaea*, were used to construct neighbour-joining, maximum parsimony (100 replicates) and maximum-likelihood phylogenetic trees. Maximum-likelihood trees of near full-length sequences were generated in ARB (Ludwig et al., 2004) using Tree-Puzzle (Schmidt et al., 2002) with the Hasegawa–Kishino–Yano model (Hasegawa et al., 1985). Shorter sequences were added to maximum-likelihood trees using the ARB parsimony insertion tool (Ludwig et al., 1998).

Quartet-puzzling (QP) reliability values are not shown at bifurcations if they are below 50%. In determining clades QP values from 90% to 100% are strongly supported; however, QP values less than 70% can also be trusted (Schmidt et al., 2002). Clades delineated here with QP values lower than 70% were analysed relative to QP support values of the other branches in the tree (Schmidt et al., 2002). Shorter sequences added to maximum-likelihood trees with the parsimony insertion tool in ARB (Ludwig et al., 1998) do not have QP support values. To minimize the number of sequences inserted by parsimony, short sequences from Einen et al. (2006), Lysnes et al. (2004a,b), Thorseth and et al. (2001), and basalt enrichment cultures were designated as operational taxonomic units (OTU, cut-off 97%). One sequence representing each OTU was inserted into the maximum-likelihood trees.

Ocean crust clades were delineated with near full-length sequences. Ocean crust clades were reported here only if all methods of phylogenetic reconstruction (parsimony, neighbour-joining and maximum-likelihood) identified the group. Sequences that did not group with clades as predicted given phylum designations were checked using the Ribosomal Database Project II Classifier (Cole et al., 2005) to verify taxonomy.

Results

Clone library construction, RFLP analysis and gene sequencing

Four basalt enrichment cultures established by M.M. Moeseneder, C.A. Di Meo-Savoie, J. Durnin, J. Josef, O.U. Mason, M.R. Fisk and S.J. Giovannoni (unpublished) were analysed by cloning, RFLP screening and sequencing. The new data in this article are from 188 archaeal and 188 bacterial 16S rRNA gene clones

from these enrichment cultures. Of the bacterial clones, 176 that contained full-length inserts were analysed by RFLP. Forty-two unique RFLP patterns were observed. One clone from each RFLP pattern was sequenced. Four sequences were determined to be chimeric; the remaining 39 clones fell into 19 unique clades. Of the archaeal clones, 170 were found to have full-length inserts and were analysed by RFLP. A total of 16 unique RFLP patterns were found. One clone from each of these was sequenced, producing 12 new archaeal sequences that passed tests for chimerism.

Phylogenetic analysis

The alignment used for phylogenetic analysis included sequences from basalt samples, basalt enrichment cultures, basalt isolates and the most similar sequences in public databases. For each clade, the most closely related sequences from cultured isolates were included in the analysis. Quartet puzzling support values were used to estimate confidence in clades. While the specific focus of this article is the phylogeny of microorganisms associated with marine basalts, other relevant data sets were incorporated into our database, such as sequences from crustal fluids (Cowen et al., 2003; Huber et al., 2006) and from iron-oxidizing bacteria capable of growth on basalt (Edwards et al., 2003).

Figure 2.1 provides an overview of the phylogenetic diversity of sequences recovered from basalt samples. Fourteen bacterial and archaeal phyla and subphyla have been reported (Figure 2.1). The most frequently reported phyla are the *Gamma-* and *Alphaproteobacteria* and the Marine Group I (MGI) Crenarchaeota. Below we

Figure 2.1. Overview of the Bacteria and Archaea associated with marine basalts.

Overview of the different bacterial and archaeal phyla and subphyla elicited by culture-independent and culture-dependent methods from marine basalts. The number of sequences in each group is shown by a colour scale.

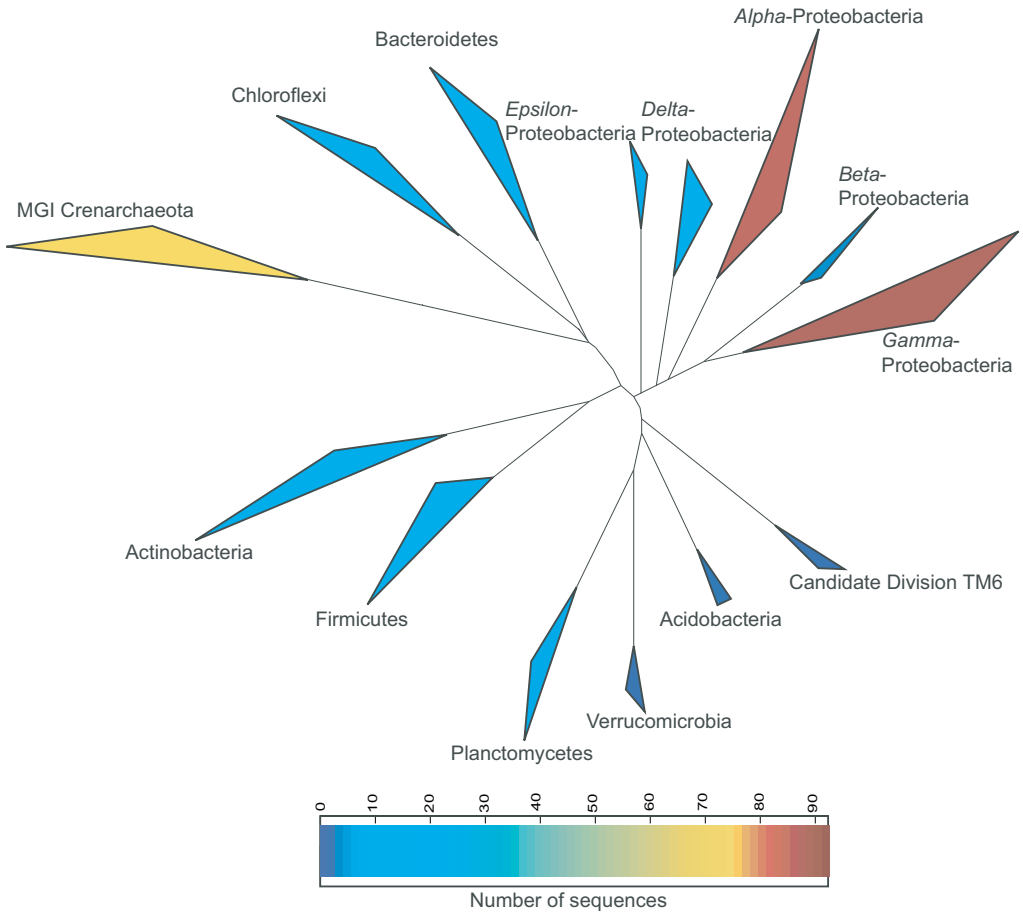

Alpha-Proteobacteria
Bacteroidetes
Chloroflexi
Epsilon-
Proteobacteria
Delta-
Proteobacteria
Beta-
Proteobacteria
MGI Crenarchaeota
Gamma-
Proteobacteria
Actinobacteria
Candidate Division TM6
Firmicutes
Acidobacteria
Verrucomicrobia
Planctomycetes
0
10
20
30
40
50
60
70
80
90
Number of sequences

Figure 2.1.

describe 11 clades that emerged with robust phylogenetic support that contain sequences exclusively from ocean crust environments.

Seven bacterial ocean crust clades (OCC) emerged from phylogenetic reconstruction: *Alphaproteobacteria* OCC I and OCC II, *Gammaproteobacteria* OCC III, *Acidobacteria* OCC IV, *Actinobacteria* OCC V, and *Verrucomicrobia* OCC VI and VII. None of the new bacterial sequences from basalt enrichment cultures were affiliated with the bacterial OCC. In contrast, several novel archaeal sequences from these enrichment cultures are members of MGI OCC. In the MGI Crenarchaeota, three *Alpha*-MGI OCC (OCC VIII–X) and one distinct MGI OCC (OCC XI) are proposed.

Bacteria

Alphaproteobacteria.

Alphaproteobacteria were frequently observed in cultivation studies and in environmental diversity surveys (Figure 2.1). Two *Alphaproteobacteria* OCC were identified (Figure 2.2). *Alphaproteobacteria* OCC I is a monophyletic clade of microorganisms in the order *Rhodobacterales*, genus *Sulfitobacter*. In the *Alphaproteobacteria* OCC I are the Mn(II)-oxidizing isolates KBB-2 and SPB: 1–4, obtained from Loihi Seamount basalts, and a sequence from sediments sampled from the western Pacific warm pool (Zeng et al., 2005) (Appendix B). *Alphaproteobacteria* OCC II consists of uncultured microorganisms from basalts collected at 9°N and from Cobb Seamount (Figure 2.2). Also in this clade are sequences from hydrothermal sediments sampled from the Mid-Atlantic Ridge (Lopez-Garcia et al., 2003) and from sediments sampled from the western Pacific

Figure 2.2. ML tree of alphaproteobacteria 16S rDNA sequences.

Maximum-likelihood phylogenetic tree of *Alphaproteobacteria* 16S rDNA sequences. The *Alphaproteobacteria* tree was constructed with 25 000 puzzling steps. An *Alphaproteobacteria* filter was used. The 16S rDNA sequence of *Shewanella frigidimarina* (U85903) was used as the outgroup. Sequences that are underlined are from ocean crust, sequences that are underlined and bold are from environmental samples of basalt, sequences that are underlined, bold and italic are from basalt enrichment cultures, and sequences that are underlined and italicized are basalt isolates. Short sequences [designated with an asterisk (*) following the GenBank accession number] were inserted using the ARB parsimony insertion tool (Ludwig et al., 1998).

Methylarcula sp. KBB-3, DQ412070
Methylarcula sp. SPB-6, DQ412076
Loktanella hongkongensis, AY600300
D3825, BECC1447a-22, EF067909
Uncultured Roseobacter 667-19, AJ294355
Bacterium K2−53A, AY345414
Uncultured Rhodobacter clone LA4-B3, AF513932
Ruegeria atlantica, D88526
Uncultured Roseobacter NAC11-3, AF245632
Acinetobacter sp. LOB-6,DQ412066
Roseobacter sp. LOB-8, DQ412059
Sulfitobacter mediterraneus, Y17387
Sulfitobacter pontiacus, Y13155
Sulfitobacter sp. KBB-2, DQ412069
Sulfitobacter sp. SPB-1, DQ412071
Sulfitobacter sp. SPB-2, DQ412072
Sulfitobacter sp. SPB-3, DQ412073
Sulfitobacter sp. SPB-4, DQ412074
Uncultured bacterium clone F46, AY375125
Marine bacterium SCRIPPS_101, AF359537
Sulfitobacter dubius, AY180102
Roseobacter sp. GAI-109, AF098494
Uncultured Rhodobacterales bacterium clone 4B4-58, DQ315619*
Jannaschia helgolandensis, AJ438157
Uncultured Rhodobacterales bacterium clone 3B4-41, DQ315618*
Tanella fryxellensis, AJ582225
Uncultured Rhodobacterales bacterium clone 2B3-19, DQ315617*
Uncultured alpha proteobacterium ODP-1155B-13, AY129892*
Marine alpha proteobacterium AS-26, AJ391187
Uncultured alpha proteobacterium ODP-1161A-536, AY129897*
Roseobacter sp. ISM, AF098495
D3825, BECC1442a-38, EF581286*
Uncultured Rhodobacterales bacterium clone 4B4-57, DQ315620*
Uncultured alpha proteobacterium ODP-1161A-528, AY129895*
Uncultured alpha proteobacterium clone 9NBGBact_18, DQ070795
Uncultured alpha proteobacterium clone R103-B61, AF449222
Agrobacterium sanguineum, AB062105
Sphingomonas terrae, D13727
Uncultured Sphingomonadales bacterium clone 4B3-53, DQ315654*
Uncultured Sphingomonadales bacterium clone 3B3-37, DQ315663*
Uncultured alpha proteobacterium ODP-1161A-534, AY129900*
Phaeospirillum fulvum, D14433
Rhodospirillum photometricum, AJ222662
Uncultured Rhizobiales bacterium clone 5B1-66, DQ315608*
Uncultured Rhodobacterales bacterium clone 5B3-70, DQ315610*
Agrobacterium kieliense, D88524
Mesorhizobium tianshanense, U71079
Uncultured Rhizobiales bacterium clone 3B1-31, DQ315607*
Uncultured Rhizobiales bacterium clone 5B3-69, DQ315612*
Fulvimarina pelagi, AY178860
Uncultured Rhizobiales bacterium clone 5B1-63, DQ315644*
Uncultured Rhizobiales bacterium clone 4B1-46, DQ315646*
Uncultured alpha proteobacterium clone MBMPE38, AJ567552
Uncultured forest soil bacterium clone DUNssu165, AY913372
Uncultured Rhizobiales bacterium clone 4B4-60, DQ315641*
Uncultured alpha proteobacterium clone JdFBGBact_41, DQ070828
Uncultured bacterium clone A56, AY373412
Rhodovibrio salinarum, M59069
Simonsiella muelleri, M59071
Uncultured alpha proteobacterium SM00-19D-303N, AY463839*
Uncultured Rhizobiales bacterium clone 5B4-72, DQ315642*
Uncultured Rhizobiales bacterium clone 4B2-49, DQ315669*
Uncultured Rhizobiales bacterium clone 3B1-32, DQ315624*
Uncultured organism clone ctg_CGOAA69, DQ395471
Uncultured alpha proteobacterium clone JdFBGBact_40, DQ070827
Alpha proteobacterium PWB3, AB106120
Uncultured alpha proteobacterium SM00-22D-296N, AY463840*
Uncultured alpha proteobacterium clone 9NBGBact_80, DQ070812
Uncultured bacterium clone B21, AY375070
Uncultured alpha proteobacterium clone JdFBGBact_66, DQ070832
Uncultured alpha proteobacterium clone AT-s3-44, AY225603
Uncultured alpha proteobacterium clone 9NBGBact_92, DQ070813
Uncultured alpha proteobacterium Arctic95B-2, AF355036
Uncultured alpha proteobacterium clone 9NBGBact_74, DQ070810
Uncultured alpha proteobacterium clone Nubeena66, AY499897
Uncultured Hyphomonas sp. clone 2B4-20, DQ315623*
Hyphomonas jannaschiana, AJ227814
Uncultured alpha proteobacterium SM00-22D-295N, AY463837*
Maricaulis maris, AJ007807
Uncultured Rhizobiales bacterium clone 5B4-71, DQ315628*
Caulobacter fusiformis, AJ007803
D3825, BECC1442a-55, EF581287*
Uncultured Rhodospirillaceae bacterium clone 1B0-5, DQ315636*
D3825, BECC1447a-90, EF581296*
Uncultured alpha proteobacterium SM00-17D-266N, AY463846*
Salinisphaera shabanensis, AJ421425
Uncultured bacterium clone BT60DS1BA2, AF365680
Shewanella frigidimarina, U85903
Alpha-Proteobacteria OCC I
Alpha-Proteobacteria OCC II
0.10

Figure 2.2.

warm pool (Zeng et al., 2005) (Appendix B). This monophyletic OCC is composed of sequences described as unclassified *Alphaproteobacteria*.

Gammaproteobacteria.

Although this subphylum was highly represented in cultivation- and non-cultivation-based analyses (Figure 2.1), only a single *Gammaproteobacteria* OCC (OCC III) emerged (Figure 2.3). This clade is comprised of environmental sequences from 9°N and from sediments above a gas hydrate at the Cascadia Margin, Oregon (Knittel et al., 2003) (Appendix B). One sequence from 9°N is taxonomically identified as a member of the order *Chromatiales*, and the remaining are unclassified environmental sequences from *Gammaproteobacteria*.

Acidobacteria.

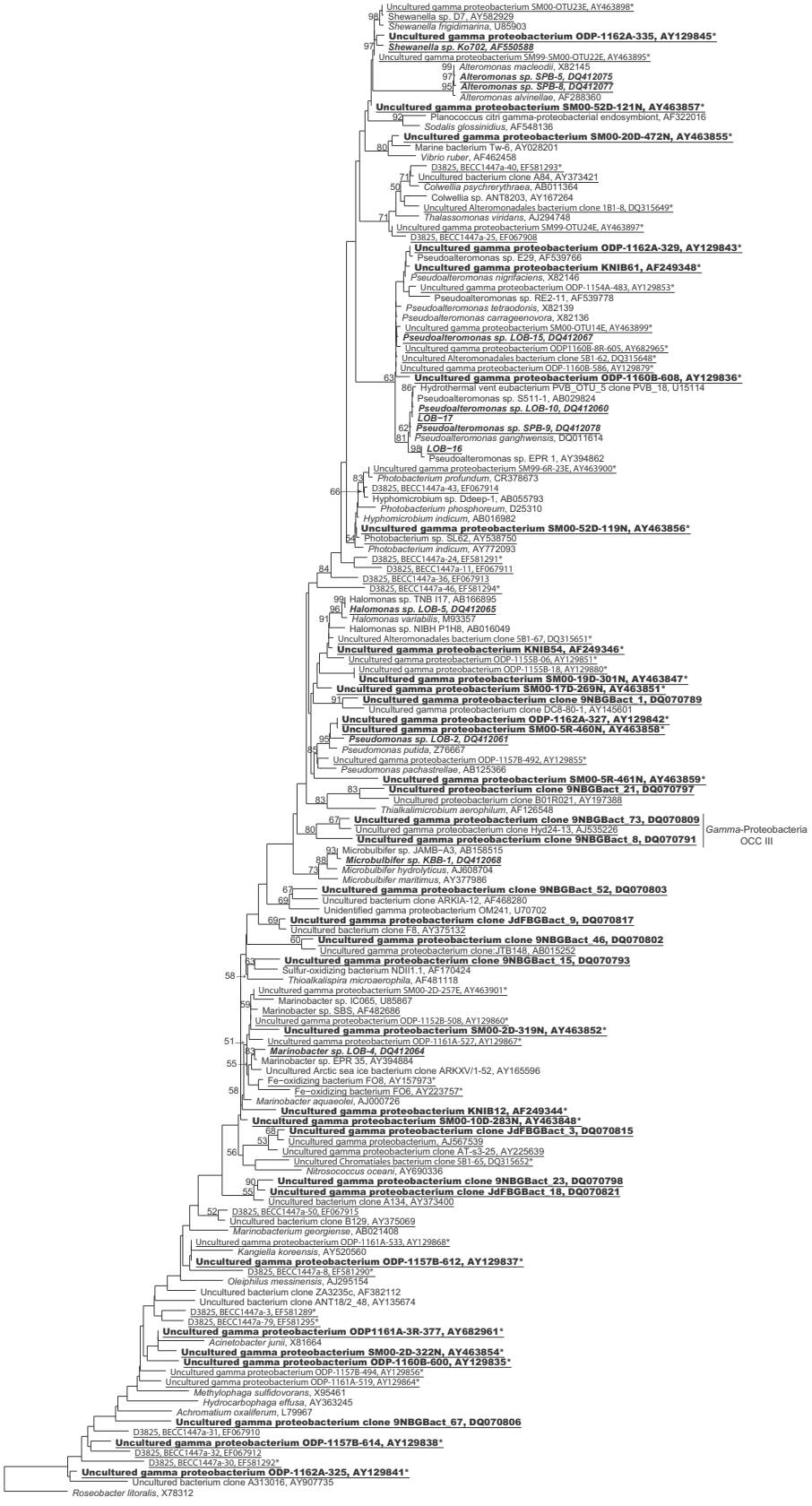
All *Acidobacteria* from basalts form a monophyletic clade (Figure 2.4). This clade, *Acidobacteria* OCC IV, is composed of uncultured, unclassified *Acidobacteria* from 9°N, Cobb Seamount, coastal marine sediments and North Sea sediments (Asami et al., 2005; Musat et al., 2006) (Appendix B).

Actinobacteria.

Actinobacteria OCC V is monophyletic clade of uncultured, unclassified *Actinobacteria* from the Southeast Indian Ridge, Mohns Ridge (Lysnes et al., 2004a) and 9°N (Figure 2.4). Also in this clade are sequences from deep-sea sediments (Li

Figure 2.3. ML tree of *Gammaproteobacteria* 16S rDNA sequences.

Maximum-likelihood phylogenetic tree of *Gammaproteobacteria* 16S rDNA sequences. The *Gammaproteobacteria* tree was constructed with 50 000 puzzling steps. A *Gammaproteobacteria* filter was used. The 16S rDNA sequence of *Roseobacter litoralis* (X78312) was used as the outgroup.


Uncultured gamma proteobacterium SM00-OTU23E, AY463898*
Shewanella sp. D7, AY582929
Shewanella frigidimarina, U85903
Uncultured gamma proteobacterium ODP-1162A-335, AY129845*
Shewanella sp. Ko702, AF550588
Uncultured gamma proteobacterium SM99-SM00-OTU22E, AY463895*
Alteromonas macleodii, X82145
Alteromonas sp. SPB-5, DQ412075
Alteromonas sp. SPB-8, DQ412077
Alteromonas alvinellae, AF288360
Uncultured gamma proteobacterium SM00-52D-121N, AY463857*
Planococcus citri gamma-proteobacterial endosymbiont, AF322016
Sodalis glossinidius, AF548136
Uncultured gamma proteobacterium SM00-20D-472N, AY463855*
Marine bacterium Tw-6, AY028201
Vibrio ruber, AF462458
D3825, BECC1447a-40, EF581293*
Uncultured bacterium clone A84, AY373421
Colwellia psychrerythraea, AB011364
Colwellia sp. ANT8203, AY167264
Uncultured Alteromonadales bacterium clone 1B1-8, DQ315649*
Thalassomonas viridans, AJ294748
Uncultured gamma proteobacterium SM99-OTU24E, AY463897*
D3825, BECC1447a-25, EF067908
Uncultured gamma proteobacterium ODP-1162A-329, AY129843*
Pseudoalteromonas sp. E29, AF539766
Uncultured gamma proteobacterium KNIB61, AF249348*
Pseudoalteromonas nigrifaciens, X82146
Uncultured gamma proteobacterium ODP-1154A-483, AY129853*
Pseudoalteromonas sp. RE2-11, AF539778
Pseudoalteromonas tetraodonis, X82139
Pseudoalteromonas carrageenovora, X82136
Uncultured gamma proteobacterium SM00-OTU14E, AY463899*
Pseudoalteromonas sp. LOB-15, DQ412067
Uncultured gamma proteobacterium ODP1160B-8R-605, AY682965*
Uncultured Alteromonadales bacterium clone 5B1-62, DQ315648*
Uncultured gamma proteobacterium ODP-1160B-586, AY129879*
Uncultured gamma proteobacterium ODP-1160B-608, AY129836*
Hydrothermal vent eubacterium PVB_OTU_5 clone PVB_18, U15114
Pseudoalteromonas sp. S511-1, AB029824
Pseudoalteromonas sp. LOB-10, DQ412060
LOB-17
Pseudoalteromonas sp. SPB-9, DQ412078
Pseudoalteromonas ganghwensis, DQ011614
LOB-16
Pseudoalteromonas sp. EPR 1, AY394862
Uncultured gamma proteobacterium SM99-6R-23E, AY463900*
Photobacterium profundum, CR378673
D3825, BECC1447a-43, EF067914
Hyphomicrobium sp. Ddeep-1, AB055793
Photobacterium phosphoreum, D25310
Hyphomicrobium indicum, AB016982
Uncultured gamma proteobacterium SM00-52D-119N, AY463856*
Photobacterium sp. SL62, AY538750
Photobacterium indicum, AY772093
D3825, BECC1447a-24, EF581291*
D3825, BECC1447a-11, EF067911
D3825, BECC1447a-36, EF067913
D3825, BECC1447a-46, EF581294*
Halomonas sp. TNB I17, AB166895
Halomonas sp. LOB-5, DQ412065
Halomonas variabilis, M93357
Halomonas sp. NIBH P1H8, AB016049
Uncultured Alteromonadales bacterium clone 5B1-67, DQ315651*
Uncultured gamma proteobacterium KNIB54, AF249346*
Uncultured gamma proteobacterium ODP-1155B-06, AY129851*
Uncultured gamma proteobacterium ODP-1155B-18, AY129880*
Uncultured gamma proteobacterium SM00-19D-301N, AY463847*
Uncultured gamma proteobacterium SM00-17D-269N, AY463851*
Uncultured gamma proteobacterium clone 9NBGBact_1, DQ070789
Uncultured gamma proteobacterium clone DC8-80-1, AY145601
Uncultured gamma proteobacterium ODP-1162A-327, AY129842*
Uncultured gamma proteobacterium SM00-5R-460N, AY463858*
Pseudomonas sp. LOB-2, DQ412061
Pseudomonas putida, Z76667
Uncultured gamma proteobacterium ODP-1157B-492, AY129855*
Pseudomonas pachastrellae, AB125366
Uncultured gamma proteobacterium SM00-5R-461N, AY463859*
Uncultured proteobacterium clone 9NBGBact_21, DQ070797
Uncultured proteobacterium clone B01R021, AY197388
Thialkalimicrobium aerophilum, AF126548
Uncultured gamma proteobacterium clone 9NBGBact_73, DQ070809
Uncultured gamma proteobacterium clone Hyd24-13, AJ535226
Uncultured gamma proteobacterium clone 9NBGBact_8, DQ070791
Gamma-Proteobacteria OCC III
Microbulbifer sp. JAMB-A3, AB158515
Microbulbifer sp. KBB-1, DQ412068
Microbulbifer hydrolyticus, AJ608704
Microbulbifer maritimus, AY377986
Uncultured gamma proteobacterium clone 9NBGBact_52, DQ070803
Uncultured bacterium clone ARKJA-12, AF468280
Unidentified gamma proteobacterium OM241, U70702
Uncultured gamma proteobacterium clone JdFBGBact_9, DQ070817
Uncultured bacterium clone F8, AY375132
Uncultured gamma proteobacterium clone 9NBGBact_46, DQ070802
Uncultured gamma proteobacterium clone JTB148, AB015252
Uncultured gamma proteobacterium clone 9NBGBact_15, DQ070793
Sulfur-oxidizing bacterium NDII1.1, AF170424
Thioalkalispira microaerophila, AF481118
Uncultured gamma proteobacterium SM00-2D-257E, AY463901*
Marinobacter sp. IC065, U85867
Marinobacter sp. SBS, AF482686
Uncultured gamma proteobacterium ODP-1152B-508, AY129860*
Uncultured gamma proteobacterium SM00-2D-319N, AY463852*
Uncultured gamma proteobacterium ODP-1161A-527, AY129867*
Marinobacter sp. LOB-4, DQ412064
Marinobacter sp. EPR 35, AY394884
Uncultured Arctic sea ice bacterium clone ARKXV/1-52, AY165596
Fe-oxidizing bacterium FO8, AY157973*
Fe-oxidizing bacterium FO6, AY223757*
Marinobacter aquaeolei, AJ000726
Uncultured gamma proteobacterium KNIB12, AF249344*
Uncultured gamma proteobacterium SM00-10D-283N, AY463848*
Uncultured gamma proteobacterium clone JdFBGBact_3, DQ070815
Uncultured gamma proteobacterium, AJ567539
Uncultured gamma proteobacterium clone AT-s3-25, AY225639
Uncultured Chromatiales bacterium clone 5B1-65, DQ315652*
Nitrosococcus oceani, AY690336
Uncultured gamma proteobacterium clone 9NBGBact_23, DQ070798
Uncultured gamma proteobacterium clone JdFBGBact_18, DQ070821
Uncultured bacterium clone A134, AY373400
D3825, BECC1447a-50, EF067915
Uncultured bacterium clone B129, AY375069
Marinobacterium georgiense, AB021408
Uncultured gamma proteobacterium ODP-1161A-533, AY129868*
Kangiella koreensis, AY520560
Uncultured gamma proteobacterium ODP-1157B-612, AY129837*
D3825, BECC1447a-8, EF581290*
Oleiphilus messinensis, AJ295154
Uncultured bacterium clone ZA3235c, AF382112
Uncultured bacterium clone ANT18/2_48, AY135674
D3825, BECC1447a-3, EF581289*
D3825, BECC1447a-79, EF581295*
Uncultured gamma proteobacterium ODP1161A-3R-377, AY682961*
Acinetobacter junii, X81664
Uncultured gamma proteobacterium SM00-2D-322N, AY463854*
Uncultured gamma proteobacterium ODP-1160B-600, AY129835*
Uncultured gamma proteobacterium ODP-1157B-494, AY129856*
Uncultured gamma proteobacterium ODP-1161A-519, AY129864*
Methylophaga sulfidovorans, X95461
Hydrocarbophaga effusa, AY363245
Achromatium oxaliferum, L79967
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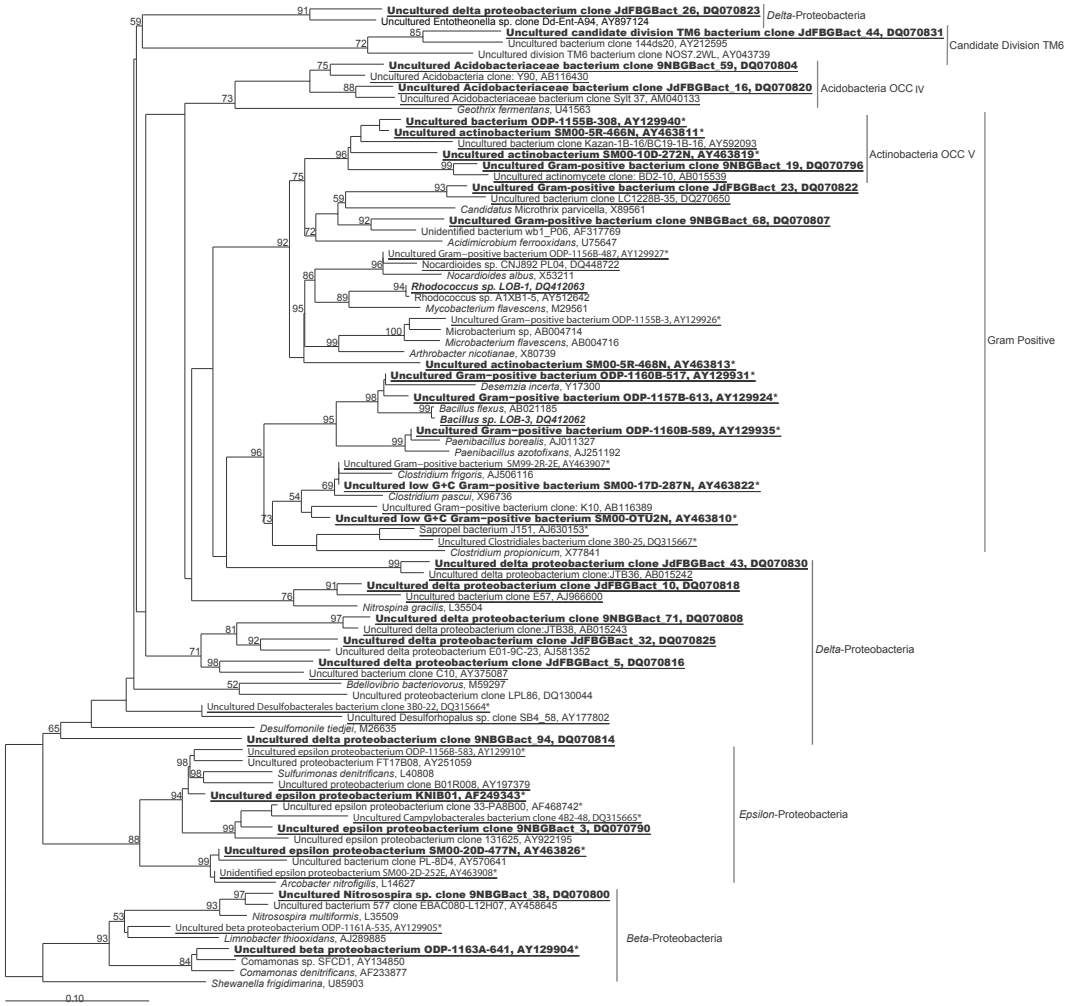
Uncultured gamma proteobacterium ODP-1157B-614, AY129838*
D3825, BECC1447a-32, EF067912
D3825, BECC1447a-30, EF581292*
Uncultured gamma proteobacterium ODP-1162A-325, AY129841*
Uncultured bacterium clone A313016, AY907735
Roseobacter litoralis, X78312
0.10

Figure 2.3.

Figure 2.4. ML tree of Gram-positive, Acidobacteria, Beta-, Delta- and Epsilonproteobacteria, and Candidate Division TM6 16S rDNA sequences.

Maximum-likelihood phylogenetic tree of Gram-positive, *Acidobacteria*, *Beta-*, *Delta-* and *Epsilonproteobacteria*, and Candidate Division TM6 16S rDNA sequences. This tree was constructed with 25 000 puzzling steps. A general *Bacteria* filter was used. The 16S rDNA sequence of *Shewanella frigidimarina* (U85903) was used as the outgroup.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Delta-Proteobacteria
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Uncultured bacterium clone 144ds20, AY212595
Uncultured division TM6 bacterium clone NOS7.2WL, AY043739
Candidate Division TM6
Uncultured Acidobacteriaceae bacterium clone 9NBGBact_59, DQ070804
Uncultured Acidobacteria clone: Y90, AB116430
Uncultured Acidobacteriaceae bacterium clone JdFBGBact_16, DQ070820
Uncultured Acidobacteriaceae bacterium clone Sylt 37, AM040133
Acidobacteria OCC IV
Geothrix fermentans, U41563
Uncultured bacterium ODP-1155B-308, AY129940*
Uncultured actinobacterium SM00-5R-466N, AY463811*
Uncultured bacterium clone Kazan-1B-16/BC19-1B-16, AY592093
Uncultured actinobacterium SM00-10D-272N, AY463819*
Uncultured Gram-positive bacterium clone 9NBGBact_19, DQ070796
Uncultured actinomycete clone: BD2-10, AB015539
Actinobacteria OCC V
Uncultured Gram-positive bacterium clone JdFBGBact_23, DQ070822
Uncultured bacterium clone LC1228B-35, DQ270650
Candidatus Microthrix parvicella, X89561
Uncultured Gram-positive bacterium clone 9NBGBact_68, DQ070807
Unidentified bacterium wb1_P06, AF317769
Acidimicrobium ferrooxidans, U75647
Uncultured Gram−positive bacterium ODP-1156B-487, AY129927*
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Nocardioides albus, X53211
Rhodococcus sp. LOB-1, DQ412063
Rhodococcus sp. A1XB1-5, AY512642
Mycobacterium flavescens, M29561
Uncultured Gram−positive bacterium ODP-1155B-3, AY129926*
Microbacterium sp, AB004714
Microbacterium flavescens, AB004716
Arthrobacter nicotianae, X80739
Uncultured actinobacterium SM00-5R-468N, AY463813*
Uncultured Gram−positive bacterium ODP-1160B-517, AY129931*
Desemzia incerta, Y17300
Uncultured Gram−positive bacterium ODP-1157B-613, AY129924*
Bacillus flexus, AB021185
Bacillus sp. LOB-3, DQ412062
Uncultured Gram−positive bacterium ODP-1160B-589, AY129935*
Paenibacillus borealis, AJ011327
Paenibacillus azotofixans, AJ251192
Uncultured Gram−positive bacterium SM99-2R-2E, AY463907*
Clostridium frigoris, AJ506116
Uncultured low G+C Gram−positive bacterium SM00-17D-287N, AY463822*
Clostridium pascui, X96736
Uncultured Gram−positive bacterium clone: K10, AB116389
Uncultured low G+C Gram−positive bacterium SM00-0TU2N, AY463810*
Sapropel bacterium J151, AJ630153*
Uncultured Clostridiales bacterium clone 3B0-25, DQ315667*
Clostridium propionicum, X77841
Gram Positive
Uncultured delta proteobacterium clone JdFBGBact_43, DQ070830
Uncultured delta proteobacterium clone:JTB36, AB015242
Uncultured delta proteobacterium clone JdFBGBact_10, DQ070818
Uncultured bacterium clone E57, AJ966600
Nitrospina gracilis, L35504
Uncultured delta proteobacterium clone 9NBGBact_71, DQ070808
Uncultured delta proteobacterium clone:JTB38, AB015243
Uncultured delta proteobacterium clone JdFBGBact_32, DQ070825
Uncultured delta proteobacterium E01-9C-23, AJ581352
Uncultured delta proteobacterium clone JdFBGBact_5, DQ070816
Uncultured bacterium clone C10, AY375087
Bdellovibrio bacteriovorus, M59297
Uncultured proteobacterium clone LPL86, DQ130044
Uncultured Desulfobacterales bacterium clone 3B0-22, DQ315664*
Uncultured Desulfonhopalus sp. clone SB4_58, AY177802
Desulfomonile tiedjei, M26635
Delta-Proteobacteria
Uncultured delta proteobacterium clone 9NBGBact_94, DQ070814
Uncultured epsilon proteobacterium ODP-1156B-583, AY129910*
Uncultured proteobacterium FT17B08, AY251059
Sulfurimonas denitrificans, L40808
Uncultured proteobacterium clone B01R008, AY197379
Uncultured epsilon proteobacterium KNIB01, AF249343*
Uncultured epsilon proteobacterium clone 33-P4B00, AF468742*
Uncultured Campylobacterales bacterium clone 4B2-48, DQ315665*
Uncultured epsilon proteobacterium clone 9NBGBact_3, DQ070790
Uncultured epsilon proteobacterium clone 131625, AY922195
Uncultured epsilon proteobacterium SM00-20D-477N, AY463826*
Uncultured bacterium clone PL-8D4, AY570641
Unidentified epsilon proteobacterium SM00-2D-252E, AY463908*
Arcobacter nitrofigilis, L14627
Epsilon-Proteobacteria
Uncultured Nitrosospira sp. clone 9NBGBact_38, DQ070800
Uncultured bacterium 577 clone EBAC080-L12H07, AY458645
Nitrosospira multiformis, L35509
Uncultured beta proteobacterium ODP-1161A-535, AY129905*
Limnobacter thiooxidans, AJ289885
Uncultured beta proteobacterium ODP-1163A-641, AY129904*
Comamonas sp. SFCD1, AY134850
Comamonas denitrificans, AF233877
Shewanella frigidimarina, U85903
Beta-Proteobacteria
0.10

Figure 2.4.

et al., 1999) and from sediments from the Kazan mud volcano, Eastern Mediterranean (S.K. Heijs, P.W.J.J. van der Wielen and L.J. Forney, unpublished) (Appendix B).

Verrucomicrobia.

Two *Verrucomicrobia* OCC were identified (Figure 2.5). *Verrucomicrobia* OCC VI is a monophyletic clade of microorganisms from 9°N basalt and anoxic marine sediments (Freitag and Prosser, 2003) (Appendix B). *Verrucomicrobia* OCC VII is composed of sequences from Cobb Seamount basalt and from deep-sea marine sediments (Li et al., 1999) (Appendix B). The *Verrucomicrobia* in these OCC were taxonomically identified as unclassified *Bacteria*.

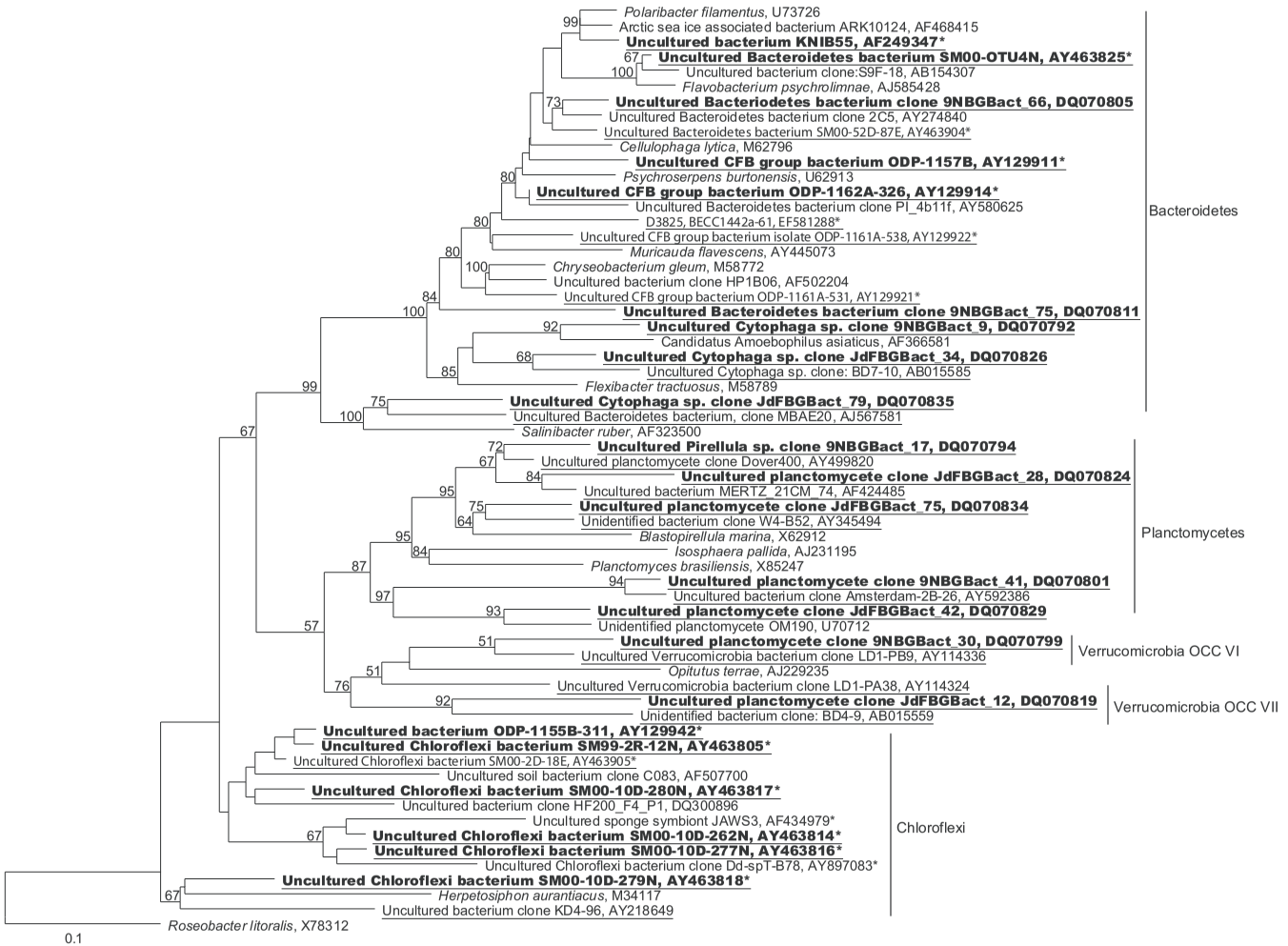
Archaea

Numerous MGI Crenarchaeota sequences were retrieved from basalts. In fact, the MGI were observed in all molecular analyses that examined the archaeal community in basalts, and were also found in enrichment cultures (Figure 2.1). Several OCC emerged in the MGI clade (Figure 2.6) that were paraphyletic with the MGI subgroups named by Massana et al. (2000; *Alpha*-, *Beta*- and *Gamma*-MGI subgroups). Within the *Alpha*-MGI, three OCC (VIII–X) emerged. *Alpha*-MGI OCC VIII includes sequences obtained from HSDP and Cobb Seamount basalts, a novel sequence from a basalt enrichment culture, and sequences from sediment sampled from the western Pacific warm pool (Zeng et al., 2005) (Figure 2.6 and Appendix C).

Alpha-MGI OCC IX is composed of sequences from 9°N, Cobb Seamount and Knipovich Ridge (Thorseth et al., 2001) basalts (Figure 2.6). This clade is the only

Figure 2.5. ML tree of Bacteroidetes, Planctomycetes, Verrucomicrobia and Chloroflexi 16S rDNA sequences.

Maximum-likelihood phylogenetic tree of *Bacteroidetes*, *Planctomycetes*, *Verrucomicrobia* and *Chloroflexi* 16S rDNA sequences. This tree was constructed with 10 000 puzzling steps. A general *Bacteria* filter was used. The 16S rDNA sequence of *Roseobacter litoralis* (X78312) was used as the outgroup.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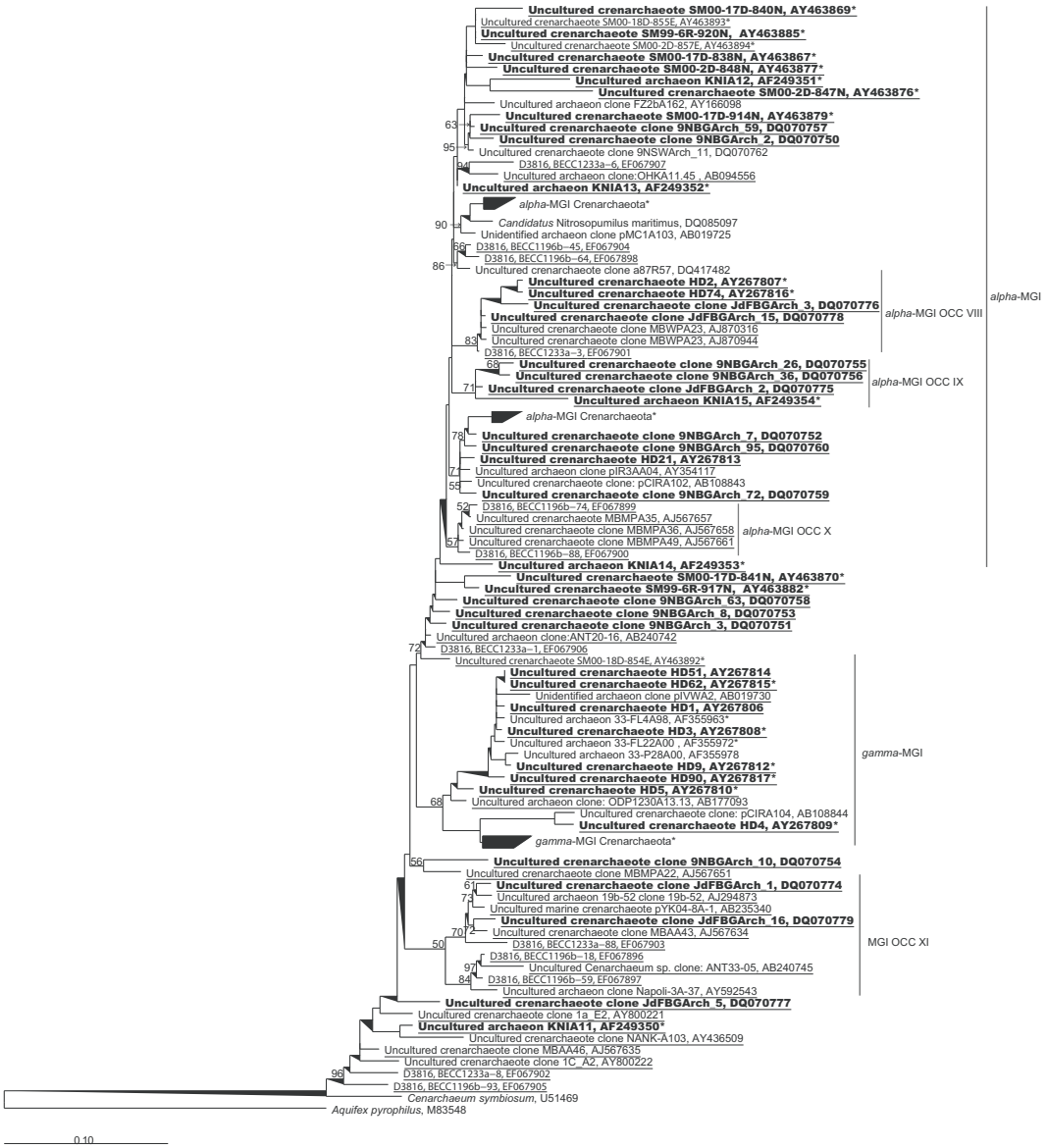
Arctic sea ice associated bacterium ARK10124, AF468415
Uncultured bacterium KNIB55, AF249347*
Uncultured Bacteroidetes bacterium SM00-OTU4N, AY463825*
Uncultured bacterium clone:S9F-18, AB154307
Flavobacterium psychrolimnae, AJ585428
Uncultured Bacteriodetes bacterium clone 9NBGBact_66, DQ070805
Uncultured Bacteroidetes bacterium clone 2C5, AY274840
Uncultured Bacteroidetes bacterium SM00-52D-87E, AY463904*
Cellulophaga lytica, M62796
Uncultured CFB group bacterium ODP-1157B, AY129911*
Psychroserpens burtonensis, U62913
Uncultured CFB group bacterium ODP-1162A-326, AY129914*
Uncultured Bacteroidetes bacterium clone PI_4b11f, AY580625
D3825, BECC1442a-61, EF581288*
Uncultured CFB group bacterium isolate ODP-1161A-538, AY129922*
Muricauda flavescens, AY445073
Chryseobacterium gleum, M58772
Uncultured bacterium clone HP1B06, AF502204
Uncultured CFB group bacterium ODP-1161A-531, AY129921*
Uncultured Bacteroidetes bacterium clone 9NBGBact_75, DQ070811
Uncultured Cytophaga sp. clone 9NBGBact_9, DQ070792
Candidatus Amoebophilus asiaticus, AF366581
Uncultured Cytophaga sp. clone JdFBGBact_34, DQ070826
Uncultured Cytophaga sp. clone: BD7-10, AB015585
Flexibacter tractuosus, M58789
Uncultured Cytophaga sp. clone JdFBGBact_79, DQ070835
Uncultured Bacteroidetes bacterium, clone MBAE20, AJ567581
Salinibacter ruber, AF323500
Uncultured Pirellula sp. clone 9NBGBact_17, DQ070794
Uncultured planctomycete clone Dover400, AY499820
Uncultured planctomycete clone JdFBGBact_28, DQ070824
Uncultured bacterium MERTZ_21CM_74, AF424485
Uncultured planctomycete clone JdFBGBact_75, DQ070834
Unidentified bacterium clone W4-B52, AY345494
Blastopirellula marina, X62912
Isosphaera pallida, AJ231195
Planctomyces brasiliensis, X85247
Uncultured planctomycete clone 9NBGBact_41, DQ070801
Uncultured bacterium clone Amsterdam-2B-26, AY592386
Uncultured planctomycete clone JdFBGBact_42, DQ070829
Unidentified planctomycete OM190, U70712
Uncultured planctomycete clone 9NBGBact_30, DQ070799
Uncultured Verrucomicrobia bacterium clone LD1-PB9, AY114336
Opitutus terrae, AJ229235
Uncultured Verrucomicrobia bacterium clone LD1-PA38, AY114324
Uncultured planctomycete clone JdFBGBact_12, DQ070819
Unidentified bacterium clone: BD4-9, AB015559
Uncultured bacterium ODP-1155B-311, AY129942*
Uncultured Chloroflexi bacterium SM99-2R-12N, AY463805*
Uncultured Chloroflexi bacterium SM00-2D-18E, AY463905*
Uncultured soil bacterium clone C083, AF507700
Uncultured Chloroflexi bacterium SM00-10D-280N, AY463817*
Uncultured bacterium clone HF200_F4_P1, DQ300896
Uncultured sponge symbiont JAWS3, AF434979*
Uncultured Chloroflexi bacterium SM00-10D-262N, AY463814*
Uncultured Chloroflexi bacterium SM00-10D-277N, AY463816*
Uncultured Chloroflexi bacterium clone Dd-spT-B78, AY897083*
Uncultured Chloroflexi bacterium SM00-10D-279N, AY463818*
Herpetosiphon aurantiacus, M34117
Uncultured bacterium clone KD4-96, AY218649
Roseobacter litoralis, X78312
0.1
Bacteroidetes
Planctomycetes
Verrucomicrobia OCC VI
Verrucomicrobia OCC VII
Chloroflexi

Figure 2.5.

Figure 2.6. ML tree of Marine Group I Crenarchaeota 16S rDNA sequences.

Maximum-likelihood phylogenetic tree of Marine Group I Crenarchaeota 16S rDNA sequences. The archaeal tree was constructed with 25 000 puzzling steps. A Crenarchaeota filter was used. Short sequences used by Massana et al. (2000) to delineate subclades were inserted using the ARB parsimony insertion tool (Ludwig et al., 1998). Sequences from Massana et al. (2000) were collapsed into their respective groups and are represented by triangles. The 16S rDNA sequence of *Aquifex pyrophilus* (M83548) was used as the outgroup.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Uncultured crenarchaeote SM00-2D-857E, AY463894*
Uncultured crenarchaeote SM00-17D-838N, AY463867*
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Uncultured archaeon KNIA12, AF249351*
Uncultured crenarchaeote SM00-2D-847N, AY463876*
Uncultured archaeon clone FZ2bA162, AY166098
Uncultured crenarchaeote SM00-17D-914N, AY463879*
Uncultured crenarchaeote clone 9NBGArch_59, DQ070757
Uncultured crenarchaeote clone 9NBGArch_2, DQ070750
Uncultured crenarchaeote clone 9NSWArch_11, DQ070762
D3816, BECC1233a–6, EF067907
Uncultured archaeon clone:OHKA11.45 , AB094556
Uncultured archaeon KNIA13, AF249352*
alpha-MGI Crenarchaeota*
Candidatus Nitrosopumilus maritimus, DQ085097
Unidentified archaeon clone pMC1A103, AB019725
D3816, BECC1196b–45, EF067904
D3816, BECC1196b–64, EF067898
Uncultured crenarchaeote clone a87R57, DQ417482
Uncultured crenarchaeote HD2, AY267807*
Uncultured crenarchaeote HD74, AY267816*
Uncultured crenarchaeote clone JdFBGArch_3, DQ070776
Uncultured crenarchaeote clone JdFBGArch_15, DQ070778
Uncultured crenarchaeote clone MBWPA23, AJ870316
Uncultured crenarchaeote clone MBWPA23, AJ870944
D3816, BECC1233a–3, EF067901
alpha-MGI OCC VIII
Uncultured crenarchaeote clone 9NBGArch_26, DQ070755
Uncultured crenarchaeote clone 9NBGArch_36, DQ070756
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alpha-MGI OCC IX
alpha-MGI Crenarchaeota*
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Uncultured crenarchaeote clone MBMPA49, AJ567661
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alpha-MGI OCC X
alpha-MGI
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Uncultured crenarchaeote SM00-18D-854E, AY463892*
Uncultured crenarchaeote HD51, AY267814
Uncultured crenarchaeote HD62, AY267815*
Unidentified archaeon clone pIVWA2, AB019730
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Uncultured archaeon 33-FL4A98, AF355963*
Uncultured crenarchaeote HD3, AY267808*
Uncultured archaeon 33-FL22A00 , AF355972*
Uncultured archaeon 33-P28A00, AF355978
Uncultured crenarchaeote HD9, AY267812*
Uncultured crenarchaeote HD90, AY267817*
Uncultured crenarchaeote HD5, AY267810*
Uncultured archaeon clone: ODP1230A13.13, AB177093
Uncultured crenarchaeote clone: pCIRA104, AB108844
Uncultured crenarchaeote HD4, AY267809*
gamma-MGI Crenarchaeota*
gamma-MGI
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Uncultured crenarchaeote clone MBMPA22, AJ567651
Uncultured crenarchaeote clone JdFBGArch_1, DQ070774
Uncultured archaeon 19b-52 clone 19b-52, AJ294873
Uncultured marine crenarchaeote pYK04-8A-1, AB235340
Uncultured crenarchaeote clone JdFBGArch_16, DQ070779
Uncultured crenarchaeote clone MBAA43, AJ567634
D3816, BECC1233a–88, EF067903
D3816, BECC1196b–18, EF067896
Uncultured Cenarchaeum sp. clone: ANT33-05, AB240745
D3816, BECC1196b–59, EF067897
Uncultured archaeon clone Napoli-3A-37, AY592543
MGI OCC XI
Uncultured crenarchaeote clone JdFBGArch_5, DQ070777
Uncultured crenarchaeote clone 1a_E2, AY800221
Uncultured archaeon KNIA11, AF249350*
Uncultured crenarchaeote clone NANK-A103, AY436509
Uncultured crenarchaeote clone MBAA46, AJ567635
Uncultured crenarchaeote clone 1C_A2, AY800222
D3816, BECC1233a–8, EF067902
D3816, BECC1196b–93, EF067905
Cenarchaeum symbiosum, U51469
Aquifex pyrophilus, M83548
0.10

Figure 2.6.

OCC that is composed entirely of sequences from basalts (Appendix C). No sequences from other ocean crust environments branch within this OCC.

Alpha-MGI OCC X is represented by novel sequences from basalt enrichment cultures and sequences from sediments sampled in the Pacific nodule province (M.X. Xu, P. Wang, F.P. Wang and X. Xiao, unpublished) (Figure 2.6 and Appendix C). MGI-OCC XI is a new subclade of the MGI Crenarchaeota (Figure 2.6). In this OCC are novel sequences from aerobic and anaerobic basalt enrichment cultures and Cobb Seamount basalt. Also in this clade are sequences from Aegean Sea sediments (M.B. Brehmer, unpublished), a chimney structure in the Southern Okinawa Trough (T. Nunoura, F. Inagaki, H. Hirayama, K. Takai and K. Horikoshi, unpublished), sediments from the Pacific nodule province (M.X. Xu, P. Wang, F.P. Wang and X. Xiao, unpublished), deep-sea mud volcanoes in the Eastern Mediterranean (S.K. Heijs, P.W.J.J. van der Wielen and L.J. Forney, unpublished) and Nankai Trough cold-seep sediments (S. Arakawa, T. Sato, Y. Yoshida, R. Usami and C. Kato, unpublished) (Appendix C).

Discussion

Distribution of basalt-associated microorganisms

Many of the OCC were detected in basalts from a variety of locations, suggesting that these clades have cosmopolitan distributions. Three clades, *Actinobacteria* OCC V (Figure 2.4), and *Alpha*-MGI OCC VIII and IX (Figure 2.6), exemplify this; they contain sequences from basalts collected from a broad geographic distribution. For example, the *Actinobacteria* OCC V sequences are from the

Southeast Indian Ridge, Mohns Ridge (Lysnes et al., 2004a) and 9°N. MGI OCC VIII and IX clade members were sampled from Cobb Seamount, 9°N, the Knipovich Ridge (Thorseth et al., 2001) and HSDP basalts. The widespread occurrence of clades that are seemingly endemic to ocean crust but were observed using various methodologies strongly suggests similarities in microbial community structure among globally distributed basalts.

Basalt endemism

Alpha-MGI OCC IX was the only ocean crust clade detected in this analysis that is composed entirely of sequences from basalts. This monophyletic clade was recovered from Cobb Seamount, 9°N and the Knipovich Ridge (Thorseth et al., 2001). It may represent an ecotype adapted to the basalt environment. The remaining OCC are composed of sequences from basalts and from sediments, suggesting that, while basalt-associated microorganisms are indigenous to ocean crust, they do not appear to be endemic to basalts, but instead are observed in both marine igneous rocks and sediments. This observation supports the conclusions of Edwards et al. (2003) who found that organisms cultivated from metalliferous sediments are able to grow on basalt glass. These results suggest that, while endoliths may have specific metabolic niches, e.g. oxidation of ferrous iron, they are not confined to a single habitat type. The remaining sequences from basalts are members of paraphyletic and polyphyletic crustal clades that include sequences from non-crustal environments, many of them *Alpha*- and *Gammaproteobacteria* (Figures 2.2 and 2.3). In these groups are

microorganisms that may occur in ocean crust but are not endemic to the crustal environment.

The ocean crust clades of the *Actinobacteria* and MGI Crenarchaeota appear to be common in ocean crust samples. The MGI Crenarchaeota were observed in five out of five studies, while the *Actinobacteria* were observed in four out of five (see Table 2.1 for study details), which is noteworthy considering that these studies employed a range of different survey methods. The MGI ocean crust clades are also unusual in that they are the only OCC represented in basalt enrichment cultures, suggesting that isolates of the MGI may be obtained using media containing basalt.

The first cultivated member of the MGI Crenarchaeota clade, *Candidatus* Nitrosopumilus maritimus, is a mesophilic, ammonium-oxidizing, chemolithoautotroph that was isolated from the substratum of an aquarium (Konneke et al., 2005). The 16S rDNA sequence of this isolate is 96–97% similar to *Alpha*-MGI OCC VIII and IX (Figure 2.6). The short evolutionary distances between *Candidatus* Nitrosopumilus maritimus and these sequences suggests that ammonium oxidation may be occurring in basalt. Nitrogen averages approximately 2 p.p.m. in basalt (Marty et al., 1995), a small amount. Therefore, an exogenous input of nitrogen would be required to support growth by this metabolism. Cowen et al. (2003), and more recently Huber et al. (2006), investigated ocean crust fluids and reported elevated levels of ammonium compared with seawater, which could support the growth of ammonium-oxidizing microorganisms. The crustal fluid microbial communities described by these studies are vastly different from the communities observed in basalts; they report only one MGI sequence (Huber et al., 2006), and no bacterial ammonium oxidizers;

therefore, a niche for ammonium-oxidizing microorganisms may exist in marine basalts.

Conclusions

Phylogenetic analysis of 16S rDNA sequences revealed that basalt endoliths are cosmopolitan in their distributions and that many are endemic to ocean crust. Some members of the MGI Crenarchaeota appear to be ecotypes adapted to the basalt environment. Members of MGI clades endemic to ocean crust have been observed in enrichment cultures, but not yet isolated. The identification of microbial clades endemic to the ocean crust, particularly members of the ubiquitous MGI Crenarchaeota, will help direct scientific research to clades of organisms that are specialists in colonizing ocean crustal habitats.

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PROKARYOTIC DIVERSITY, DISTRIBUTION, AND PRELIMINARY INSIGHTS INTO THEIR ROLE IN BIOGEOCHEMICAL CYCLING IN MARINE BASALTS

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Abstract

We used molecular techniques to analyze basalts of varying ages that were collected from the East Pacific Rise, 9 °N, from the rift axis of the Juan de Fuca Ridge, and from neighboring seamounts. Cluster analysis of 16S rDNA Terminal Restriction Fragment Polymorphism data revealed that basalt endoliths are distinct from seawater and that communities clustered, to some degree, based on the age of the host rock. This age-based clustering suggests that alteration processes may affect community structure. Cloning and sequencing of bacterial and archaeal 16S rRNA genes revealed twelve different phyla and sub-phyla associated with basalts. These include the Gemmatimonadetes, Nitrospirae, the candidate phylum SBR1093 in the Bacteria, and in the Archaea Marine Benthic Group B, none of which have been previously reported in basalts. We delineated novel ocean crust clades in the gamma-Proteobacteria, Planctomycetes, and Actinobacteria that are composed entirely of basalt associated microflora, and may represent basalt ecotypes. Finally, microarray analysis of functional genes in basalt revealed that genes coding for previously unreported processes such as carbon fixation, methane-oxidation, methanogenesis, and nitrogen fixation are present, suggesting that basalts harbor previously unrecognized metabolic diversity. These novel processes could exert a profound influence on ocean chemistry.

Introduction

Oceanic basalts are one of the most abundant rock types on Earth, covering upwards of 60% of the Earth's surface. These rocks typically have high permeabilities, which enables infiltration and circulation of large quantities of seawater (Fisher, 1998; Fisher and Becker, 2000). The rock-seawater interaction results in a significant flux of energy and solutes between basalt crust and the overlying seawater (Fisher, 1998). Recent quantitative analyses revealed that basalts harbor 6×10^5 to 4×10^6 and 3×10^6 to 1×10^9 cells per g rock (Einen et al., 2008; Santelli et al., 2008). In fact, Einen et al. (2008) suggested that the total number of microorganisms present in ocean crust exceeds the number present in seawater. These observations raise intriguing questions about the role that microorganisms play in biogeochemical cycling in basalts. Biological alteration of basalt by microorganisms has been the focus of numerous studies, with compelling evidence suggesting that they do play a part in this process (Thorseth et al., 1995; Giovannoni et al., 1996; Fisk et al., 1998; Torsvik et al., 1998; Furnes and Staudigel, 1999; Furnes et al., 2001; Banerjee and Muehlenbachs, 2003; Fisk et al., 2003; Furnes et al., 2004).

Alteration, whether abiotic or biotic, intrinsically changes the chemistry and mineralogy of the rock. For example, alteration of reactive primary minerals to secondary minerals changes the chemical milieu in which endolithic microorganisms reside. These changes may result in shifts in the microbial community. Analysis of prokaryotic communities associated with marine basalts revealed that several clades appear to be cosmopolitan in their distribution, as they are associated with globally distributed basalts, (Mason et al., 2007; Santelli et al., 2008) regardless of rock age

and degree of alteration. The ubiquity of certain clades, such as the alpha-Marine Group I ocean crust clade IX delineated by Mason et al. (2007), regardless of the age of the host rock, suggests that overall basalt microflora do not change on a temporal scale. However, Lysnes et al., (2004) reported that basalts of varying ages support different microbial phyla and sub-phyla. For example, the Actinobacteria were associated with older basalts, but were absent in recently erupted material. Therefore, certain clades may, in fact, respond to alteration processes, which could, for example, affect the available electron donors and acceptors.

Certain microbial taxa may be associated with rocks of varying ages, as suggested by Lysnes et al., (2004); however, it is unclear what factors contribute to this habitat specificity. Fresh basalts are ~ 8 % wt FeO and 2 % wt Fe_2O_3. The increasing oxidation of reduced iron with time could lead to a shift in the microbial community from oxidizers to reducers. In fact, Edwards et al. (2003b) demonstrated that chemolithoautrophic, iron-oxidizing alpha- and gamma-proteobacteria isolated from sulfides and metalliferous sediments are able to grow on basalt glass. These isolates are capable of using oxygen and nitrate as electron acceptors. This metabolic diversity would be requisite as basalt alteration progresses and the *in situ* redox conditions change.

Alternately, the reduced iron available to iron-oxidizing prokaryotes, may become hydrated during fluid-rock interactions. This reaction can evolve hydrogen (Proskurowski et al., 2008), which can serve as an electron donor for numerous microorganisms including methanogens and sulfate reducers. This abiotic hydrogen production would serve not only as an electron donor for methanogenesis and sulfate

reduction, but also for Fischer-Tropsch type reactions, from which methane is evolved (Proskurowski et al., 2008). In fact, volatiles, such as methane, have been shown to sustain microbial populations at the Lost City Hydrothermal Field (Kelley et al., 2005). Abiotically produced hydrocarbons, such as methane, as has been reported at the Lost City Hydrothermal Field (LCHF) (Proskurowski et al., 2008) could provide carbon and energy for growth in the basaltic environment.

While the geological characteristics of basalts, such as the availability of FeO for microbial iron oxidation, discussed above, do provide some insight into potential metabolic function in this environment, examination of the *in situ* metabolic diversity of prokaryotes by cultivation efforts is limited to one study. Templeton et al. (2005) isolated Mn-oxidizing, heterotrophic Bacteria from Loihi Seamount. Thus, there is a need to circumvent the lack of cultured microorganisms using a molecular approach to determine metabolic diversity in basalts. GeoChip is a molecular tool that does not rely on cultivation based methods to assay for functional diversity. Specifically, it is a functional gene microarray that has 24 243 oligonucleotide probes covering >10 000 genes in >150 functional groups involved in nitrogen, carbon, sulfur, and phosphorus cycling (He et al., 2007). GeoChip can provide significant insight into metabolic potential in a given environment, such as in marine basalts.

In this study we used terminal restriction fragment polymorphism (T-RFLP), cloning and sequencing, and microarray analysis of functional genes to 1) assess successional changes in the microbial communities associated with basalts of varying ages and from different geographical locations, 2) examine species composition and

distribution, and 3) determine potential metabolic function in basalts by examining functional genes.

Our analyses revealed that rock age, or degree of alteration, may play a role in community succession. Additionally, here we report previously unrecognized phyla in basalts and several novel ocean crust clades of microorganisms that may represent basalt specialists. Finally, examination of functional genes in basalt revealed the genetic potential for several novel metabolic processes. This analysis provides insight into biogeochemical cycling in this ocean crust environment.

Material and methods

Sample collection

Glassy pillow basalts were collected from areas of low (or no) sediment accumulation using the *DSV Alvin* on two separate cruises to East Pacific Rise (9 °N) and to the CoAxial segment of the Juan de Fuca Ridge (JdF) and neighboring seamounts (Table 3.1). Basalt samples were collected and placed inside a collection box, or "biobox" which was designed to prevent sample exposure to ambient seawater during the ascent to the surface. Prior to the dive, the box was filled with either 0.2 μm filtered seawater or sterile Millipore water. During Alvin's descent, residual airspace was replaced with seawater that passed through 0.2 μm filters embedded in the lid. The biobox also had a mechanism for injecting a 20 ml suspension of fluorescent microspheres into the box after the sample was collected and the lid was closed. The microspheres were used to track the incursion of seawater into the rock after the rock was collected. The biobox volume (16 liters) allowed for several liters of ambient deep

Table 3.1. Basalt samples from the EPR and JdF.

Basalt samples collected from the East Pacific Rise and the Juan de Fuca Ridge.

TABLE 3.1. Basalt samples collected from the East Pacific Rise and the Juan de Fuca Ridge.

Alvin dive[a]	Date	Latitude	Longitude	Depth (m)	Dive feature[b]	Age[c]	Molecular analyses
East Pacific Rise (EPR) R/V Atlantis Voyage 7 Leg 3							
D3713C	10/19/01	09° 50.80' N	104° 17.63' W	2493	base of Q vent	1991 eruption	T-RFLP
D3716A	10/22/01	09° 50.30' N	104° 17.51' W	2499	axial caldera	"	T-RFLP
D3718B	10/24/01	09° 50.78' N	104° 17.58' W	2493	north of Q vent	"	T-RFLP, cloning & sequencing[d]
D3719D	10/25/01	09° 50.78' N	104° 17.58' W	2496	near M vent	"	T-RFLP
D3720R	10/26/01	09° 50.78' N	104° 17.58' W	2498	near TY vent	"	T-RFLP
D3721D	10/27/01	09° 50.79' N	104° 17.59' W	2495	near Q vent	"	T-RFLP
D3721E	10/27/01	09° 50.79' N	104° 17.59' W	2496	near Q vent	"	T-RFLP
Juan de Fuca Ridge (JdF) R/V Atlantis Voyage 7 Leg 19[e]							
D3815F	8/05/02	45° 59.50' N	129° 56.59 W	2135	Helium Basin	<100 Ka	T-RFLP, cloning & sequencing[f]
D3816F-1,2	8/06/02	46° 31.34' N	129° 29.94 W	2653	Co-Axial Rift	10-170 Ka	T-RFLP
D3823M	8/19/02	46° 41.95' N	130° 55.94 W	1909	Cobb Seamount	3.3 Ma	T-RFLP, cloning & sequencing[d]
D3826U	8/23/02	46° 31.16' N	129° 34.92 W	2409	lava flow	1993 eruption	T-RFLP

[a]The Alvin dive number and suffix is the sample identifier.

[b]The 9 °N samples were collected from the area of the 1991 eruption (Haymon et al., 1993) and were 11 years old at the time of collection. The region of EPR vents is described in Fornari and Embley (1995).

[c]The ages of Juan de Fuca samples from Helium Basin and Co-Axial Rift, were inferred from seafloor spreading rate and distance from the ridge axis. Age of the Cobb Seamount sample from Desonie and Duncan (1990). D3826U was collected from the 1993 lava flow (Embley et al., 2000).

[d]D3718B and D3823M clones were analyzed and presented in Mason et al. (2007) and are only included in dendrograms in this study if they are part of novel clades delineated here, or are closely related to clones from D3815F.

[e]Thin sections were made for basalts from all JdF dives.

[f]D3815F clones are presented in this study.

seawater to be collected with the basalts. Once on deck, the samples were removed from the biobox using sterile (flamed) tongs and placed into separate freezer bags. Samples were immediately frozen at -80ºC and remained frozen until shore-based analyses. To control for deep-sea planktonic organisms that may have found their way into fractures and pores in the basalt samples, the biobox water was filtered and the filters were frozen and analyzed along with the basalts (see below).

Nucleic acid extraction from the basalt samples

For molecular analyses all rock sample handling and all extraction steps were performed in a sterile laminar flow hood. Ceramic tumbling vessels, chisels, mortar and pestles were baked at 220 °C for at least 24 hours. The outer rock surface was removed by tumbling the rock several times for 20 minutes, replacing with sterile grit each time. The glassy rind was pared away with a chisel and/or sterile rock splitter. Approximately 1 cm^3 was powdered with a tungsten mortar and pestle, and 2 ml of powder was used in each extraction. Two control DNA extractions, to which either 2 ml of the grit from the last tumbling step or no rock or grit material were added, were used to assess contamination introduced by the tumbling steps or from the DNA extraction reagents, respectively. DNA extractions were carried out according to Fisk et al. (2003).

DNA extraction from the filtered biobox water samples

At least 12 L of biobox water from each dive was filtered through a 142 mm 0.2 μm Supor filter (Pall Gelman Laboratory, Ann Arbor, MI) in a polycarbonate filter

holder (Geotech Environmental Equipment, Inc. Denver, CO) connected to a peristaltic pump. Filters were immediately preserved in 5 ml of sucrose lysis buffer (20 mM EDTA, 400 mM NaCl, 0.75 M sucrose, 50 mM Tris·HCl, pH 9.0) and stored at -80 °C. Total community nucleic acids were extracted from the filters according to (Giovannoni et al., 1990).

Terminal-restriction fragment length polymorphism (T-RFLP) analysis

T-RFLP analysis was used to compare the archaeal and bacterial communities from several rock and corresponding biobox seawater samples according to Moeseneder et al. (1999) with few modifications. The archaeal 16S rRNA genes were amplified using the primers Arch20F (DeLong et al., 1999) and Arch915R (Stahl and Amann, 1991), with the forward primer 5' end-labeled with phosphoramidite fluorochrome 5-carboxy-fluorescein (6-FAM) and the reverse primer labeled with 5-hexachlorofluorescein (5-HEX). Fifty PCR cycles were necessary to amplify archaeal DNA, while a semi-nested approach was required to amplify bacterial 16S rRNA genes from nearly all of the basalts, with primers 27F-B (5'-AGRGTTYGATYMTGGCTCAG) and 1492RY (5'-GGYTACCTTGTTACGACTT) modified from (Lane, 1991) used in the initial PCR reaction (30 cycles), and primers 27F-B-[FAM] and 1391R (Lane, 1991) used in the second reaction (20 cycles). Only the forward strand of this PCR product was labeled for the T-RFLP analysis. For both archaeal and bacterial amplifications, three replicate PCR reactions (50 µl) for each DNA sample contained final concentrations of the following: 1µl of DNA extract; 1% (v/v) PCR buffer (+ NH_4SO_4; MBI Fermentas, Hanover, MD); 0.2 mM each

deoxynucleotide triphosphate; 0.2 μM each primer; 2 mM $MgCl_2$ (MBI Fermentas); 1.2 mg ml^{-1} bovine serum albumin (non-acetylated, SIGMA); 1% (wt/v) PVP (polyvinylpyrrolidone); 2.5 U Taq polymerase (MBI Fermentas). PCR cycling consisted of denaturation at 94 °C for 1.5 min, annealing at 55 °C for 1.5 min, and extension at 72 °C for 1.5 min. The filtered biobox seawater samples from each dive were also analyzed using the same cycling conditions as the basalts, except the number of cycles was reduced to 30.

PCR products (50 ng) were digested with 10 units of enzyme for 6 hrs at 37 ºC with each of three separate restriction enzymes: AluI, BsuRI (HaeIII), and Hin6I (HhaI) (MBI Fermentas). Samples were run on an ABI 3100 (Applied Biosystems, Inc. (ABI), Foster City, CA). The fingerprint patterns for the rock and seawater communities were compared according to Moeseneder et al. (1999); however, only peaks longer than 70 bp in length were included in the analysis. Data were standardized by inclusion of peaks that represented >1% of the total peak height for each fingerprint and were then converted to binary matrices. Binary data were analyzed by the unweighted pair group with mathematical averages (UPGMA) method in PAUP* (Phylogenetic Analysis Using Parsimony *(and Other Methods)) version 4.0 b10 (Swofford, 1998) using the site distance matrix method of Nei and Li (1979) according to Moeseneder et al. (1999).

Thin sections

All samples used in this study were seafloor basalts with quenched glass exteriors and fine-grained interiors. Singly polished thin sections were prepared from

cross sections of the exterior of D3815F, D3816F, D3823M, and D3826U basalt samples of different ages from Juan de Fuca Ridge and nearby Cobb Seamount. These samples represent recently erupted basalts, to older, more weathered basalts.

PCR amplification and cloning of prokaryotic 16S rRNA genes

Data from the UPGMA analysis was used to select three basalt samples that differed in age and community structure (D3718B, 9 °N EPR, D3815F and D3823M Juan de Fuca) for cloning and sequencing of archaeal and bacterial 16S rRNA genes. The archaeal communities were amplified according to the PCR conditions described above for T-RFLP analysis, except the primers were not fluorescently labeled. To amplify archaeal 16S rDNA from D3815F and bacterial 16S rDNA from D3718B the semi-nested approach described above was used. Amplification of bacterial 16S rDNA from D3823M did not require a semi-nested approach. Corresponding seawater samples from dives 3718B and 3823M were also cloned for comparison. PCR reactions (50 µl vol) were cloned into the pGEM®-T Easy vector (Promega Corp., Madison, WI). Clone libraries were constructed and screened according to the methods of Vergin et al. (2001). Briefly, clones were assigned to clone families based upon shared patterns for two separate restriction digests. Digested PCR products were resolved on a 3% agarose gel. One clone from each unique RFLP pattern was sequenced using an ABI 3730 capillary sequencer. Full-length sequences were obtained for clones representing each phylotype. Chimeric sequences were identified with the CHECK_CHIMERA program (Maidak et al., 1997; Maidak et al., 1999) and Mallard (Ashelford et al., 2006).

Phylogenetic analysis

The phylogenies of microorganisms from D3718B and D3823M were extensively reviewed in Mason et al. (2007). Clones from these libraries are presented here in phylogenetic dendrograms only if they are part of novel ocean crust clades delineated here, or if they are highly similar to clones from D3815F. However, clones from all libraries were analyzed during phylogenetic reconstruction. Phylogenetic analyses and clade delineations were carried out according to Mason et al. (2007), using the Greengenes database (DeSantis et al., 2006).

Nucleotide sequence accession numbers

The 16S rRNA gene sequences for the archaeal and bacterial clones were submitted to the GenBank database and have been assigned the following accession numbers: DQ070750 to DQ070835 (D3718B and D3823M). Sequences for D3815F have been submitted to GenBank, but have not yet been assigned accession numbers.

Functional genes

D3815F was selected for functional gene analysis because it had several clades that have not been previously reported from this environment, particularly the archaeal Marine Benthic Group B. We hypothesized that this diversity of species would be mirrored in the diversity of functional genes. Second, thin sections of this sample showed textures that suggest bioalteration; therefore, analysis of functional genes in this sample would provide insight into the biological processes that may result in these textural features. Functional genes were assayed for using the GeoChip 2.0 (He et al.,

2007) microarray following previously described methods (Wu et al., 2006; He et al., 2007). Briefly, DNA from D3815F was amplified in triplicate using a Templiphi 500 amplification kit (Amersham Biosciences, Piscataway, NJ) with modifications as previously described (Wu et al., 2006). Amplified DNA was fluorescently labeled with Cy5. Hybridizations were performed using a HS4800Pro Hybridization Station (TECAN, US, Durham, NC) overnight at 42 °C. Microarrays were scanned using a ProScanArray (PerkinElmer, Waltham, MA). Images were then analyzed using ImaGene 6.0 (BioDiscovery, El Segundo, CA) to designate the identity of each spot and to determine spot quality. Data was processed as described by Wu et al. (2006). Briefly, raw data from Imagene was analyzed using a GeoChip data analysis pipeline. A signal to noise ratio of ≥ 3 was considered a positive signal. A positive signal in at least 1/3 of the probes for a particular gene (minimum of 2 probes) was required for a gene to be considered positive. Each gene had 1, 2, or 3 probes per array based on the number of probes available meeting the criteria described by He et al. (2007)

Results and Discussion

T-RFLP

UPGMA cluster analysis of T-RFLP data revealed that the archaeal and bacterial communities were distinct from deep seawater communities (Figure 3.1). Further, there was striking congruency in the UPGMA clustering patterns for the four oldest JdF samples. These old samples, ranging from a few thousand to about three million years in age, clustered together, while the younger basalts from 9 °N (from an eruption in 1991) clustered with one JdF sample of a similar age (D3826U, from the

Figure 3.1. UPGMA analysis of T-RFLP fingerprint patterns for the Bacteria and Archaea.

UPGMA analysis of T-RFLP fingerprint patterns for the Bacterial (left) and Archaeal (right) communities recovered from basalts (above the dashed line) and background seawater from 9N and JdF (below the dashed). Older basalts (> 20 years) from JdF are indicated with an underline. All 9 °N samples are less than 20 years. Sample numbers indicate Alvin dive number and location: 9N is 9°N on the East Pacific Rise and JdF is Juan de Fuca Ridge and Cobb Seamount.

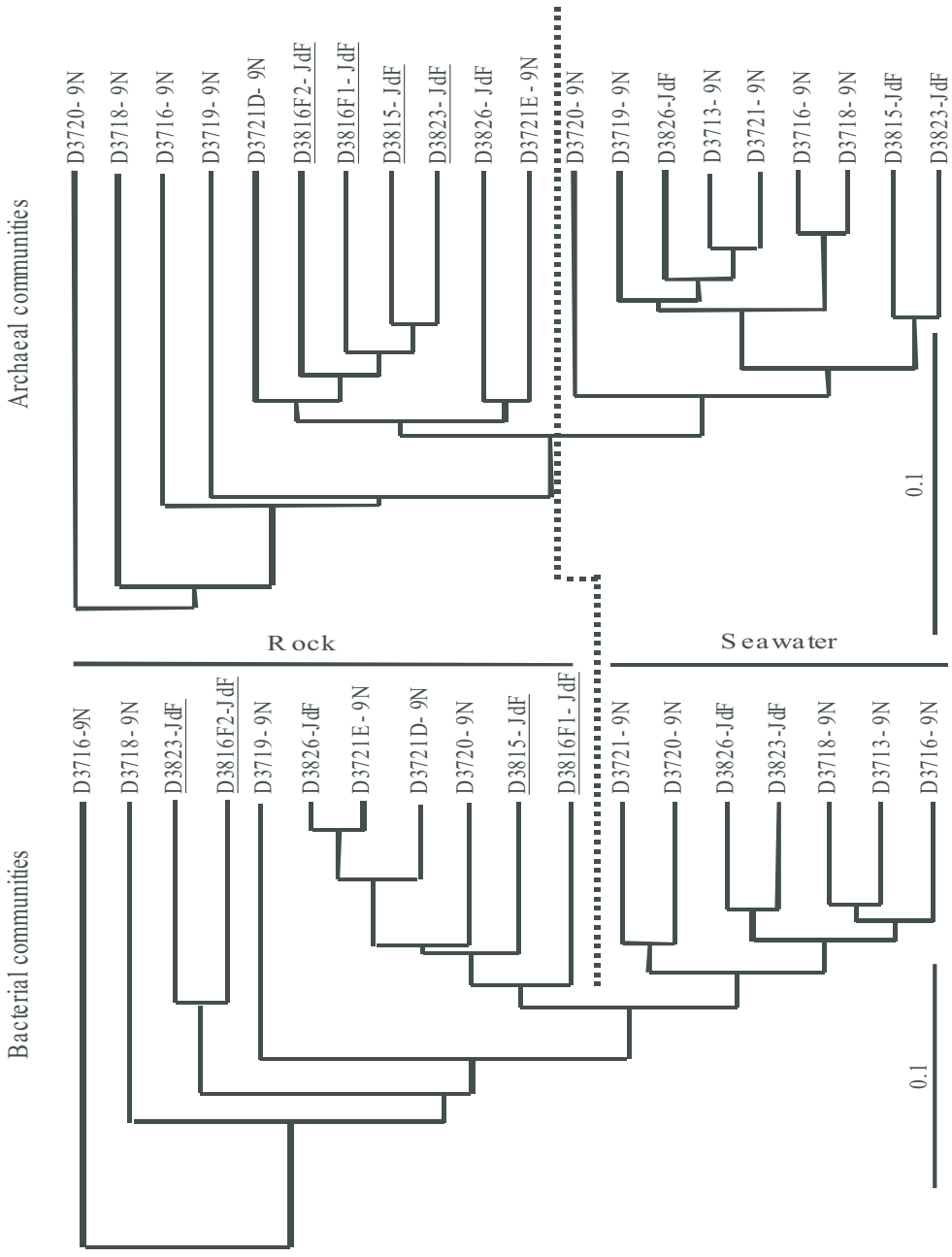
Archaeal communities
D3720-9N
D3718-9N
D3716-9N
D3719-9N
D3721D-9N
D3816F2-JdF
D3816F1-JdF
D3815-JdF
D3823-JdF
D3826-JdF
D3721E-9N
D3720-9N
D3719-9N
D3826-JdF
D3713-9N
D3721-9N
D3716-9N
D3718-9N
D3815-JdF
D3823-JdF
0.1
Rock
Seawater
Bacterial communities
D3716-9N
D3718-9N
D3823-JdF
D3816F2-JdF
D3719-9N
D3826-JdF
D3721E-9N
D3721D-9N
D3720-9N
D3815-JdF
D3816F1-JdF
D3721-9N
D3720-9N
D3826-JdF
D3823-JdF
D3718-9N
D3713-9N
D3716-9N
0.1

Figure 3.1.

1993 lava flow). This clustering is evidence that there are differences in microbial communities present in recently erupted basalts compared to older, more weathered rocks. The observed clustering is supported, to some degree, by phylogeny. For example, the Planctomycetes ocean crust clade XIV members (see below) are from recently erupted to medium-aged basalts. Overall, however, there is distinct overlap in the microbial communities regardless of rock age. For example, the basalt specific ocean crust clade presented here, such as the gamma-Proteobacteria ocean crust clade XII, is composed of microorganisms from young, fresh basalts to 3.3 Ma year old basalts. This pattern suggests that basalt microflora are largely associated with rocks of varying ages, but that a minority may reside in, for example, younger, less altered rocks to the exclusion of older, more weathered rocks. This finding is consistent with that of Lysnes et al. (2004), who reported that specific bacterial species are found only in rocks of a certain age.

Basalt alteration

Analysis of thin sections revealed that irregular alteration textures attributed to biological processes increase in prevalence with increasing rock age (Figure 3.2). Few bioalteration textures were observed in D3826 (1993 eruption) (Figure 3.2). Bioalteration textures increased in prevalence in older rocks, D3815F (< 0.1 Ma) and D3816 (< 0.17 Ma), with the most found in the Cobb Seamount basalt (3.3 Ma) (Figure 3.2). As rocks age, several factors could result in changes that affect the microbial communities, such as changing rock permeability, weathering of primary minerals, and oxidation state of the rocks. These alteration processes, concomitant

Figure 3.2. Photomicrographs of singley polished thin sections.

Photomicrographs of singley polished thin sections of four samples from our dive sites. (A) Alvin dive 3826U on the 1993 flow. One arrow indicates a smooth alteration front without dark secondary minerals, suggestive of abiotic alteration. A second arrow indicates bubble-like alteration along a fracture, a style of alteration not seen in other basalts. (B) Alvin dive 3815F near the top of the scarp west of Helium Basin on the east side of Axial Seamount. Arrows indicate where irregular alteration has started to develop along fractures. (C) Sample from Alvin dive 3816F from a lava flow 6 km east of Juan de Fuca rift axis. Irregular alteration and dark secondary minerals suggest that biotic alteration is occurring along fractures. The width of alteration zones around fractures (white bar) are larger than seen in D3826U and D3185F. (D) Sample from Alvin dive 3823M. Alteration zones around some fractures (white bar) are wider than seen in other samples.

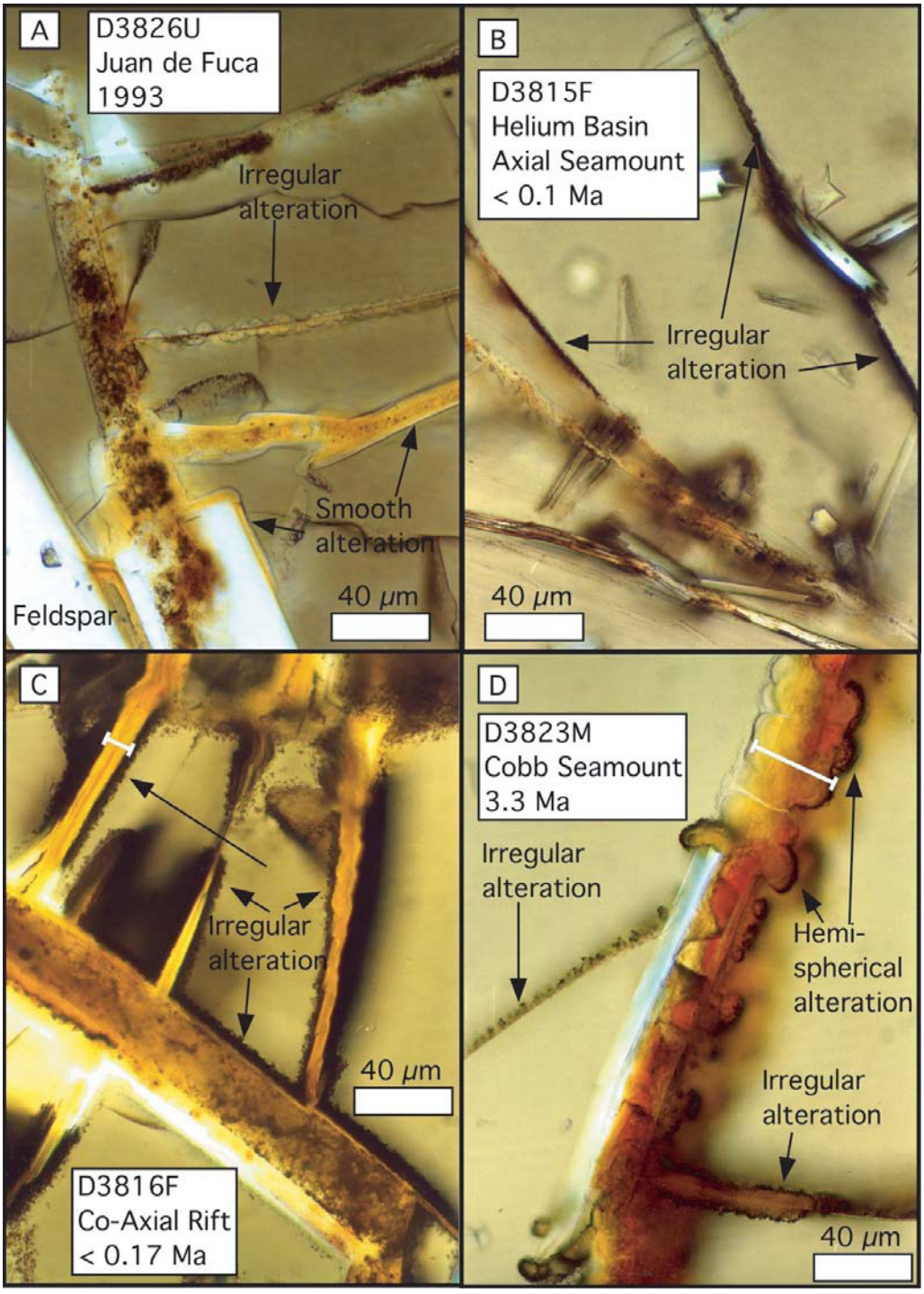
A
D3826U
Juan de Fuca
1993
Irregular
alteration
Smooth
alteration
Feldspar
40 μm
B
D3815F
Helium Basin
Axial Seamount
< 0.1 Ma
Irregular
alteration
40 μm
C
Irregular
alteration
40 μm
D3816F
Co-Axial Rift
< 0.17 Ma
D
D3823M
Cobb Seamount
3.3 Ma
Irregular
alteration
Hemi-
spherical
alteration
Irregular
alteration
40 μm

Figure 3.2.

with the clustering based on rock age in the UPGMA analysis, suggests that some intrinsic properties in the rock affects the microbial community structure. Both the clustering and the increase in bioalteration textures with age are evidence that alteration processes, whether biotic or abiotic, may serve to drive community succession.

Phylogenetic analysis

In our basalt samples we used cloning and sequencing of 16S rDNA and observed Gemmatimonadetes, Nitrospirae, SBR1093, and in the Archaea the Marine Benthic Group B (Figures 3.3, 3.4, and 3.5). None of these clades have been previously reported in basalt samples. Additionally, microorganisms in the alpha-, delta-, and gamma-Proteobacteria, Acidobacteria, Actinobacteria, Bacteroidetes, and Planctomycetes in the bacterial domain are reported (Figures 3.3 and 3.4). The most prevalent microorganisms were Proteobacteria (56%), the majority of which were gamma- (25%), alpha- (15%), and delta- (13%), followed by the Bacteroidetes (10%), Actinobacteria (9%), Planctoymcetes (7%), Acidobacteria (6%), Verrucomicrobia (3%), and Gemmatimonadetes (3%). The remaining clades were observed in a single rock sample.

Our observations are consistent with those reported by Santelli et al. (2008) who analyzed basalts from the East Pacific Rise and from Hawaii and found 68%/66% (EPR%/Hawaii%) Proteobacteria, 8%/5% Planctomycetes, 7%/8% Actinobacteria, 4%/1% Bacteroidetes, 3%/4% Acidobacteria, and 2%/2% Verrucomicrobia. The similarity in bacterial communities associated with basalts from a broad geographic

Figure 3.3. ML phylogenetic tree of Proteobacteria.

Maximum-likelihood phylogenetic tree of proteobacterial 16S rRNA gene sequences from basalt samples. The Proteobacteria tree was constructed with 25,000 puzzling steps. A general Bacteria filter was used. The 16S rDNA sequence of *Aquifex pyrophilus* (M83548) was used as the outgroup (not shown).

97% D3823M, Uncultured alpha proteobacterium clone JdFBGBact_66, DQ070832
Uncultured alpha proteobacterium clone AT-s3-44 , AY225603
64% Uncultured bacterium clone P9X2b8H08, EU491319
Uncultured bacterium clone P9X2b2F07, EU491336
D3718B, Uncultured alpha proteobacterium clone 9NBGBact_92, DQ070813
69% D3718B, Uncultured alpha proteobacterium clone 9NBGBact_80 , DQ070812
58% Uncultured bacterium clone B21, AY375070
70% D3815F, Uncultured alpha-Proteobacterium clone 29,
54% Uncultured bacterium clone EPR4059-B2-Bc8, EU491580
62% Uncultured bacterium clone EPR4059-B2-Bc21, EU491532
Defluvicoccus vanus, AF179678
95% D3823M, Uncultured alpha proteobacterium clone JdFBGBact_40, DQ070827
90% D3815F, Uncultured alpha-Proteobacterium clone 8,
Uncultured alpha proteobacterium clone LC1-25, DQ289899.1, 0.00
74% Uncultured bacterium, clone #7−3, AB250582
83% D3815F, Uncultured alpha-Proteobacterium clone 79,
Uncultured alpha proteobacterium, clone MBMPE50, AJ567563
D3823M, Uncultured Hyphomicrobium sp. clone JdFBGBact_73, DQ070833
91% Uncultured alpha proteobacterium clone LC1-35, DQ289905
70% D3815F, Uncultured alpha-Proteobacterium clone 39,
Uncultured bacterium clone AKAU3476, DQ125515
Rhizobium leguminosarum, AY509899
Rhodospirillum photometricum, AJ222662.1, 1.9607, 1428
95% Hyphomicrobium methylovorum, Y14307.1, 0.0000, 1420
94% Sphingomonas paucimobilis, U37337.1, 0.0000
D3823M, Uncultured gamma proteobacterium clone JdFBGBact_18, DQ070821
Uncultured bacterium clone P0X4b3G10, EU491426
64% Uncultured bacterium clone P0X4b2A01, EU491461
59% Uncultured bacterium clone EPR3968-O8a-Bc43, EU491710
92% D3718B, Uncultured gamma proteobacterium clone 9NBGBact_23 , DQ070798
64% D3718B, Uncultured gamma proteobacterium clone 9NBGBact_67, DQ070806
51% D3823M, Uncultured gamma proteobacterium clone JdFBGBact_3, DQ070815
D3815F, Uncultured gamma-Proteobacterium clone 14,
78% Uncultured gamma proteobacterium, clone MBMPE13, AJ567539
76% Uncultured bacterium clone P0X4b2A10, EU491466
Uncultured bacterium clone LC1446B-88, DQ270631
53% Uncultured Alteromonas sp., clone: IG-1-15, AB262378
67% D3815F, Uncultured gamma-Proteobacterium clone 21,
97% Alteromonas sp. AS−30, AJ391191
Alteromonas macleodii, AJ319537
70% Uncultured bacterium clone H6, DQ328618
97% Uncultured bacterium clone CV107, DQ499327
D3815F, Uncultured gamma-Proteobacterium clone 23,
72% Uncultured bacterium clone MSB-2H6, EF125462
76% D3815F, Uncultured gamma-Proteobacterium clone 11,
Uncultured bacterium, clone NB−04, AB117708
52% Uncultured bacterium, clone E77, AJ966605
55% Uncultured gamma proteobacterium, clone AT−s80, AY225635
95% D3815F, Uncultured gamma-Proteobacterium clone 6,
Bathymodiolus thermophilus gill symbiont, M99445
66% 75% Uncultured delta proteobacterium, clone JT58−30, AB189349
59% D3815F, Uncultured delta-Proteobacterium clone 17,
99% Uncultured delta proteobacterium clone Sva0853, AJ240985
100% Uncultured delta proteobacterium, clone JTB36, AB015242
100% D3823M, Uncultured delta proteobacterium clone JdFBGBact_43, DQ070830
Unidentified bacterium, clone BD3−7, AB015549
81% D3823M, Uncultured delta proteobacterium clone JdFBGBact_10, DQ070818
55% D3815F, Uncultured delta-Proteobacterium clone 4,
53% Uncultured bacterium, clone E57, AJ966600
87% Nitrospina gracilis, L35504
Uncultured bacterium clone EPR3967-O2-Bc7, EU491815
98% Uncultured delta proteobacterium clone G10_WMSP1, DQ450812
57% Uncultured bacterium #0319-6G20, AF234131
D3815F, Uncultured delta-Proteobacterium clone 19,
61% Geopsychrobacter electrodiphilus, AY187303
65% Desulfuromonas alkaliphilus, DQ309326
Geobacter sulfurreducens, AE017180
97% D3823M, Uncultured delta proteobacterium clone JdFBGBact_5, DQ070816
89% Uncultured bacterium clone P9X2b2C05, EU491278
Uncultured bacterium, clone C10, AY375087
Alpha-Proteobacteria
Ocean Crust Clade II
Alpha-Proteobacteria
Gamma-Proteobacteria
Ocean Crust Clade XII
Gamma-Proteobacteria
Delta-Proteobacteria

Figure 3.3.

Figure 3.4. ML phylogenetic tree of bacterial 16S rDNA sequences.

Maximum-likelihood phylogenetic tree of Actinobacteria, Cyanobacteria, Bacteroidetes, Planctomycetes, Gemmatimonadetes, Acidobacteria, Nitrospirae, and SBR1093 16S rRNA gene sequences from basalt samples. The Bacteria tree was constructed with 25,000 puzzling steps. A general Bacteria filter was used. The 16S rDNA sequence of *Aquifex pyrophilus* (M83548) was used as the outgroup (not shown).

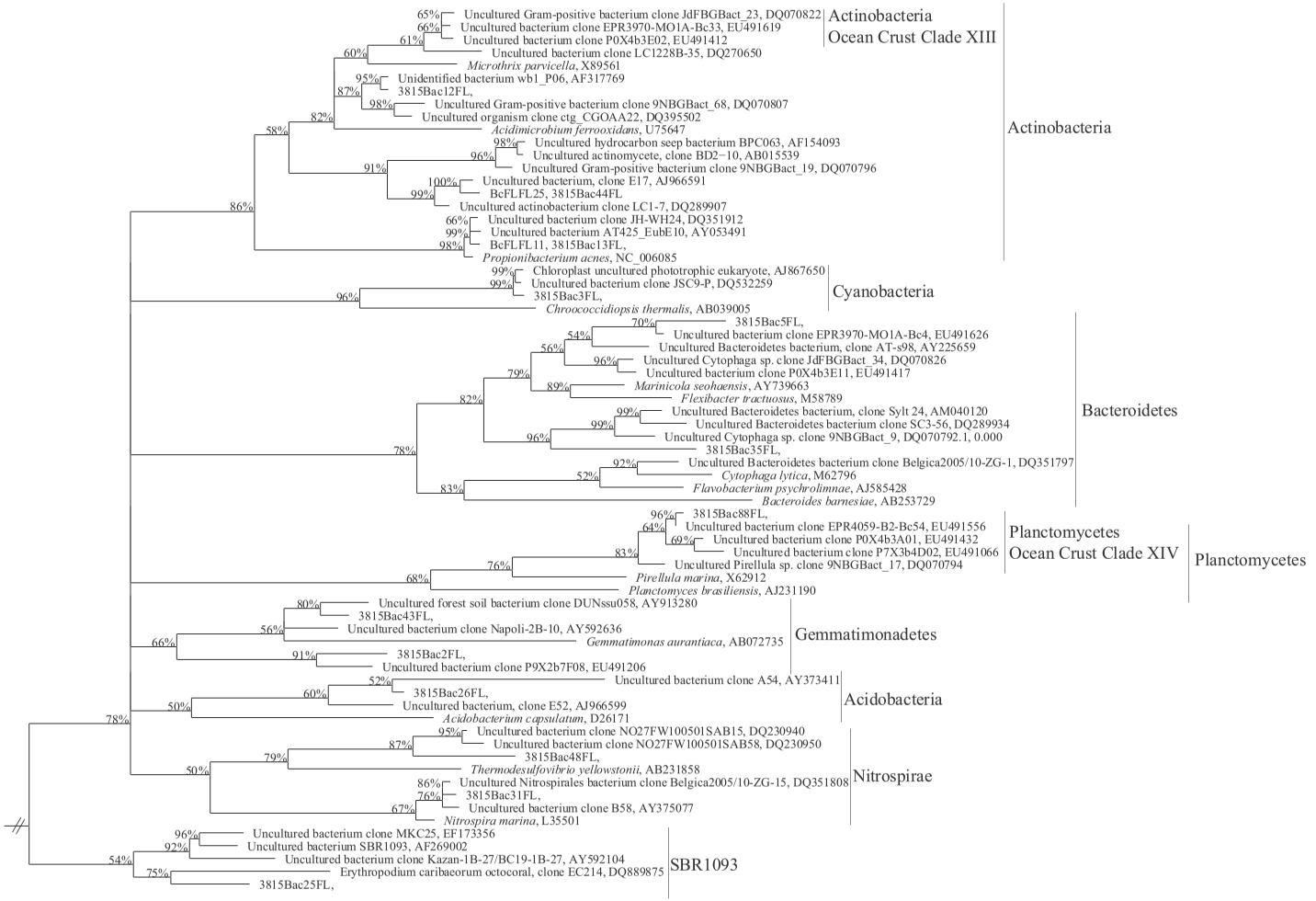
Uncultured Gram-positive bacterium clone JdFBGBact_23, DQ070822
Uncultured bacterium clone EPR3970-MO1A-Bc33, EU491619
Uncultured bacterium clone P0X4b3E02, EU491412
Actinobacteria
Ocean Crust Clade XIII
Uncultured bacterium clone LC1228B-35, DQ270650
Microthrix parvicella, X89561
Unidentified bacterium wb1_P06, AF317769
3815Bac12FL,
Uncultured Gram-positive bacterium clone 9NBGBact_68, DQ070807
Uncultured organism clone ctg_CGOAA22, DQ395502
Acidimicrobium ferrooxidans, U75647
Uncultured hydrocarbon seep bacterium BPC063, AF154093
Uncultured actinomycete, clone BD2−10, AB015539
Uncultured Gram-positive bacterium clone 9NBGBact_19, DQ070796
Uncultured bacterium, clone E17, AJ966591
BcFLFL25, 3815Bac44FL
Uncultured actinobacterium clone LC1-7, DQ289907
Uncultured bacterium clone JH-WH24, DQ351912
Uncultured bacterium AT425_EubE10, AY053491
BcFLFL11, 3815Bac13FL,
Propionibacterium acnes, NC_006085
Actinobacteria
Chloroplast uncultured phototrophic eukaryote, AJ867650
Uncultured bacterium clone JSC9-P, DQ532259
3815Bac3FL,
Cyanobacteria
Chroococcidiopsis thermalis, AB039005
3815Bac5FL,
Uncultured bacterium clone EPR3970-MO1A-Bc4, EU491626
Uncultured Bacteroidetes bacterium, clone AT-s98, AY225659
Uncultured Cytophaga sp. clone JdFBGBact_34, DQ070826
Uncultured bacterium clone P0X4b3E11, EU491417
Marinicola seohaensis, AY739663
Flexibacter tractuosus, M58789
Uncultured Bacteroidetes bacterium, clone Sylt 24, AM040120
Uncultured Bacteroidetes bacterium clone SC3-56, DQ289934
Uncultured Cytophaga sp. clone 9NBGBact_9, DQ070792.1, 0.000
3815Bac35FL,
Uncultured Bacteroidetes bacterium clone Belgica2005/10-ZG-1, DQ351797
Cytophaga lytica, M62796
Flavobacterium psychrolimnae, AJ585428
Bacteroides barnesiae, AB253729
Bacteroidetes
3815Bac88FL,
Uncultured bacterium clone EPR4059-B2-Bc54, EU491556
Uncultured bacterium clone P0X4b3A01, EU491432
Uncultured bacterium clone P7X3b4D02, EU491066
Uncultured Pirellula sp. clone 9NBGBact_17, DQ070794
Planctomycetes
Ocean Crust Clade XIV
Pirellula marina, X62912
Planctomyces brasiliensis, AJ231190
Planctomycetes
Uncultured forest soil bacterium clone DUNssu058, AY913280
3815Bac43FL,
Uncultured bacterium clone Napoli-2B-10, AY592636
Gemmatimonas aurantiaca, AB072735
3815Bac2FL,
Uncultured bacterium clone P9X2b7F08, EU491206
Gemmatimonadetes
Uncultured bacterium clone A54, AY373411
3815Bac26FL,
Uncultured bacterium, clone E52, AJ966599
Acidobacterium capsulatum, D26171
Acidobacteria
Uncultured bacterium clone NO27FW100501SAB15, DQ230940
Uncultured bacterium clone NO27FW100501SAB58, DQ230950
3815Bac48FL,
Thermodesulfovibrio yellowstonii, AB231858
Uncultured Nitrospirales bacterium clone Belgica2005/10-ZG-15, DQ351808
3815Bac31FL,
Uncultured bacterium clone B58, AY375077
Nitrospira marina, L35501
Nitrospirae
Uncultured bacterium clone MKC25, EF173356
Uncultured bacterium SBR1093, AF269002
Uncultured bacterium clone Kazan-1B-27/BC19-1B-27, AY592104
Erythropodium caribaeorum octocoral, clone EC214, DQ889875
3815Bac25FL,
SBR1093

Figure 3.4.

distribution suggests cosmopolitan distributions of these clades, which is in agreement with findings presented by Mason et al. (2007) and Santelli et al. (2008).

Phylogenetic reconstruction revealed three novel ocean crust clades composed entirely of microorganisms associated with basalt. These new clades are the gamma-Proteobacteria ocean crust clade XII (Figure 3.3), Actinobacteria ocean crust clade XIII (Figure 3.4), and Planctomycetes ocean crust clade XIV (Figure 3.4). These clades are comprised of Bacteria sampled from Juan de Fuca (this study), East Pacific Rise, 9 °N (this study and Santelli et al., 2008) and Hawaiian (Santelli et al., 2008) basalts. These cosmopolitan basalt clades may represent ecotypes of Bacteria that are specifically adapted to this environment.

Cloning and sequencing of Archaeal 16S rDNA determined that Marine Benthic Group B (MBGB) were present in basalts (Figure 3.5). This is the first report of this clade in this environment, as previous studies that examined the archaeal communities in basalts revealed only Marine Group I Crenarchaeota (MGI) (Thorseth et al., 2001; Fisk et al., 2003; Lysnes et al., 2004; Mason et al., 2007). Recently, quantitative analyses of the microbial communities in basalts revealed that Archaea comprise 4-12% (Santelli et al., 2008) and a maximum of 0.02% (Einen et al., 2008) of the prokaryotic communities. While these estimates are disparate they do reveal that Archaea are a minor component in the overall microbial communities that reside in basalt. Although Archaea are less prevalent they are ubiquitous in basalts and have been reported in all studies that assayed for their presence (Thorseth et al., 2001; Fisk et al., 2003; Lysnes et al., 2004; Mason et al., 2007; Einen et al., 2008; Santelli et al., 2008). Further, as discussed previously a clade of Marine Group I Archaea appear to

Figure 3.5. ML phylogenetic tree of archaeal 16S rDNA sequences.

Maximum-likelihood phylogenetic tree of archaeal 16S rRNA gene sequences from basalt samples. The Archaea tree was constructed with 25,000 puzzling steps. A general Archaea filter was used. The 16S rDNA sequence of *Aquifex pyrophilus* (M83548) was used as the outgroup (not shown).

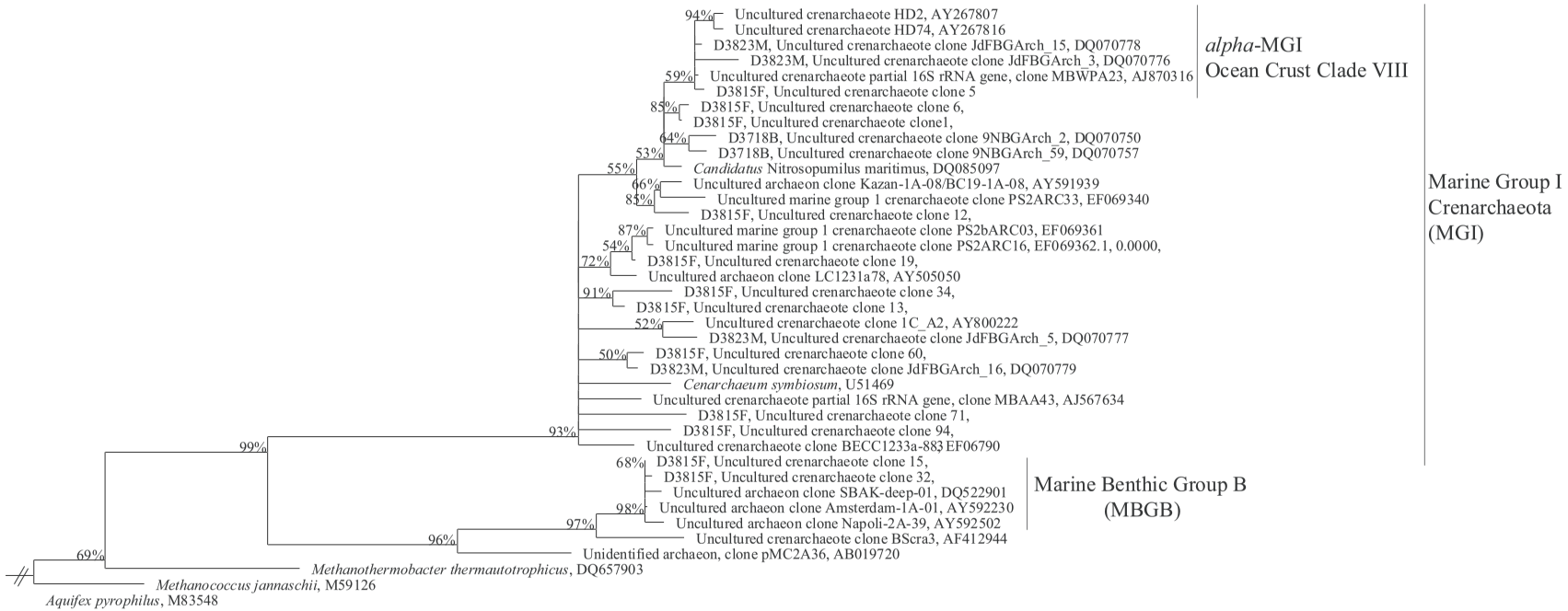
94%
Uncultured crenarchaeote HD2, AY267807
Uncultured crenarchaeote HD74, AY267816
D3823M, Uncultured crenarchaeote clone JdFBGArch_15, DQ070778
D3823M, Uncultured crenarchaeote clone JdFBGArch_3, DQ070776
59%
Uncultured crenarchaeote partial 16S rRNA gene, clone MBWPA23, AJ870316
D3815F, Uncultured crenarchaeote clone 5
85%
D3815F, Uncultured crenarchaeote clone 6,
D3815F, Uncultured crenarchaeote clone1,
64%
D3718B, Uncultured crenarchaeote clone 9NBGArch_2, DQ070750
53%
D3718B, Uncultured crenarchaeote clone 9NBGArch_59, DQ070757
55%
Candidatus Nitrosopumilus maritimus, DQ085097
66%
Uncultured archaeon clone Kazan-1A-08/BC19-1A-08, AY591939
85%
Uncultured marine group 1 crenarchaeote clone PS2ARC33, EF069340
D3815F, Uncultured crenarchaeote clone 12,
87%
Uncultured marine group 1 crenarchaeote clone PS2bARC03, EF069361
54%
Uncultured marine group 1 crenarchaeote clone PS2ARC16, EF069362.1, 0.0000,
72%
D3815F, Uncultured crenarchaeote clone 19,
Uncultured archaeon clone LC1231a78, AY505050
91%
D3815F, Uncultured crenarchaeote clone 34,
D3815F, Uncultured crenarchaeote clone 13,
52%
Uncultured crenarchaeote clone 1C_A2, AY800222
D3823M, Uncultured crenarchaeote clone JdFBGArch_5, DQ070777
50%
D3815F, Uncultured crenarchaeote clone 60,
D3823M, Uncultured crenarchaeote clone JdFBGArch_16, DQ070779
Cenarchaeum symbiosum, U51469
Uncultured crenarchaeote partial 16S rRNA gene, clone MBAA43, AJ567634
D3815F, Uncultured crenarchaeote clone 71,
93%
D3815F, Uncultured crenarchaeote clone 94,
99%
Uncultured crenarchaeote clone BECC1233a-883, EF06790
68%
D3815F, Uncultured crenarchaeote clone 15,
D3815F, Uncultured crenarchaeote clone 32,
Uncultured archaeon clone SBAK-deep-01, DQ522901
98%
Uncultured archaeon clone Amsterdam-1A-01, AY592230
97%
Uncultured archaeon clone Napoli-2A-39, AY592502
96%
Uncultured crenarchaeote clone BScra3, AF412944
Unidentified archaeon, clone pMC2A36, AB019720
69%
Methanothermobacter thermautotrophicus, DQ657903
Methanococcus jannaschii, M59126
Aquifex pyrophilus, M83548
alpha-MGI
Ocean Crust Clade VIII
Marine Group I
Crenarchaeota
(MGI)
Marine Benthic Group B
(MBGB)

Figure 3.5.

be endemic to basalt (Mason et al., 2007). This habitat specificity and global distribution indicates that some Archaea, while less abundant than Bacteria, are particularly adapted to life in basalt and likely play a role in biogeochemical cycling.

Functional genes

GeoChip (He et al., 2007) microarray analysis of functional genes in basalt sample D3815F revealed the presence of genes coding for metabolic processes previously unrecognized in this environment. In this analysis a total of 604 probes of the 24 243 total probes present on GeoChip were positive. Specifically, we found genes coding for carbon fixation, methane production and oxidation, nitrogen fixation, ammonium oxidation, nitrate and nitrite reduction, dissimilatory sulfate reduction, and iron reduction (see Appendix D for a complete list).

Here we report genes coding for carbon fixation. Basalts lacking a sediment layer are considered to be an oligotrophic, low carbon environment (Edwards et al., 2003a), thus carbon cycling in this habitat is particularly significant. The oligotrophic nature of this environment suggests that carbon fixation would be paramount in this habitat. In fact, chemolithoautotrophic processes in marine subsurface ridge flank hydrothermal environments have been theoretically shown to provide energy that could result in significant microbial biomass ($\sim 1 \times 10^{12}$ g C yr^{-1}) (Bach and Edwards, 2003). Therefore, chemolithoautrophic processes occurring *in situ* could serve to underpin a basalt hosted biosphere. One such process is methanogenesis, where hydrogen can serve as the electron donor to reduce carbon dioxide, evolving methane. During fluid-rock interactions when the basalt minerals olivine and pyroxene react

with water, hydrogen may be evolved (Proskurowski et al., 2008). Thus the requisite electron donor may be present as a result of this abiotic reaction.

Here we report that genes coding for methanogenesis are present in basalt. Methanogens have not been reported in molecular analyses of Archaea in basalts conducted to date (Thorseth et al., 2001; Fisk et al., 2003; Lysnes et al., 2004; Mason et al., 2007). However, Lysnes et al., (2004) reported that methane was evolved in enrichment cultures inoculated with marine basalts. Although the Marine Benthic Group B clade currently lacks a cultured representative (Knittel et al., 2005) they are frequently associated with environments dominated by methane, methanogens, and methanotrophs (Knittel et al., 2005; Kendall and Boone, 2006; Kendall et al., 2007). The role of this clade in the environment is unknown, but it is plausible that they are involved in methane biogeochemical cycling. Although no known methanogens were observed in our study the diversity of *mcr* genes in conjunction with a clade typically observed in methane rich environments suggests that this metabolic process may be occurring in basalts and could provide a fixed carbon source.

Abiotic hydrogen would serve not only as an electron donor for methanogenesis but also for Fischer-Tropsch type (FTT) reactions, from which methane is evolved (Proskurowski et al., 2008). Abiotic methane along with the methane resulting from biological processes could serve as a carbon and energy source for heterotrophic processes. In fact, we found genes coding for methane oxidation. Methane production and consumption could mean that basaltic crust is either a source or a sink for methane, which would have a direct impact on the overlying hydrosphere.

As discussed above, basalts are not carbon replete. Similarly they are composed of only a small amount of nitrogen, averaging approximately 2 ppm (Marty et al., 1995). Therefore, the detection of genes coding for nitrogen fixation is intriguing. Cowen et al. (2003), and more recently Huber et al. (2006), investigated ocean crust fluids and reported elevated levels of ammonium compared to seawater. Cowen et al. (2003) suggested that nitrogen fixation may serve as the source of this excess ammonium. Mehta et al. (2005) attributed nitrogen fixation in crustal fluids and in deep seawater to non-methanogenic Archaea, which are the only known archaeal nitrogen fixers. In that study, *nifH* genes were detected in crustal fluids. Nitrogen-fixation may also be taking place in the host rocks themselves given the presence of *nifH* genes in our basalt sample.

Nitrogen fixation could augment the low nitrogen concentrations in basalts and may ultimately support ammonium oxidizing microorganisms. This hypothesis is supported by the presence of genes that code for ammonium oxidation in our basalt sample. As reported by Mason et al. (2007) (see Figures 3 and 4), basalt sequences similar to *Nitrosococcus oceani* (89% similar) and *Nitrosospira multiformis* (96% similar), both of which are known ammonium-oxidizing microorganisms (Watson, 1965; Watson et al., 1971), were derived from basalts from Juan de Fuca and 9 °N (this study), and Mohns Ridge (Einen et al., 2006). Thus phylogenetic and functional gene analyses both suggest that ammonium oxidation may be occurring in basalts.

Further, basalt clones closely related to the nitrite oxidizing *Nitrospina gracilis* (91-94% similar) and *Nitrospira marina* (95-96% similar) (Watson and Waterbury, 1971; Tal et al., 2003) were found (Figures 3.3 and 3.4), suggesting that nitrite

oxidation, the second step in nitrification, may be occurring in basalts. This observation could not be confirmed using GeoChip; however, because genes coding for nitrite oxidation are not present on the gene chip.

Nitrification could provide the substrate for both denitrification and anaerobic ammonium oxidation (anammox), both of which lead to loss of nitrogen (Lam et al., 2007). In fact, we found numerous genes coding for nitrate and nitrite reduction; therefore, it is likely that denitrification is occurring in this environment. Recently, Edwards et al., (2003b) demonstrated that chemolithoautotrophic iron-oxidizing Bacteria are able to grow on basalt glass using nitrate as the electron acceptor. Whether anaerobic ammonium oxidation is taking place in basalts remains unclear. Although Planctomycetes have been reported in basalts, including the novel ocean crust clade Planctomycetes XIV delineated here, microorganisms closely related to known anammox Bacteria, such as *Kuenenia stuttgartiensis*, (77% similar to basalt associated microorganisms), have not been detected. Therefore, it is unclear if this process is important in considering nitrogen loss from the basalt layer. Our data does suggest; however, that nitrogen could be lost from marine crust by denitrification processes.

In addition to genes coding for denitrification processes, we also detected genes coding for iron-reduction and dissimilatory sulfate reduction in basalt. Together, these genes suggest that anaerobic respiration may be occurring in basalt. The presence of genes that code for aerobic respiration (e.g. ammonium oxidation) in the same sample indicates that aerobic and anaerobic processes may occur

simultaneously on a small spatial scale, suggesting, perhaps that microniches are occupied by prokaryotes in basalt.

Conclusion

Basalts from Juan de Fuca, neighboring seamounts, and 9 °N, EPR harbor cosmopolitan microorganisms that are distinct from seawater prokaryotes. Several novel ocean crust clades composed only of microorganisms from basalts suggest that some Bacteria are specifically adapted to this ocean crust environment. Our analysis of geochemically important functional genes revealed the potential for several metabolic processes not known to be occurring in basalts, particularly carbon fixation, methanogenesis, methane oxidation, nitrogen fixation and denitrification. Our data suggests that basalts not only harbor a diversity of broadly distributed microbial species, but also unexpected metabolic diversity. Future studies should utilize culture-dependent and -independent methods to analyze biogeochemical cycling in basalts to better understand the biological processes in this vast subsurface environment and how these processes ultimately affect ocean chemistry.

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HYDROCARBON-UTILIZING PROKARYOTES IN A NOVEL DEEP SUBSURFACE OCEAN CRUST ENVIRONMENT

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Abstract

Crustal fluids emanating from vents in the Lost City Hydrothermal Field (LCHF; 30 °N, 42 °W, Atlantis Massif) are enriched in hydrogen which is attributed to serpentinization reactions (Proskurowski et al., 2008). This reaction produces an environment conducive to abiotic hydrocarbon synthesis (Proskurowski et al., 2008). Methane and other low-molecular-weight carbon compounds from LCHF appear to have formed abiotically by Fischer-Tropsch type reactions (Proskurowski et al., 2008). Volatiles, such as methane have been shown to sustain microbial populations at the LCHF (Kelley et al., 2001). Here, we show that deep subsurface gabbroic samples (50-1300 meters below the seafloor), with varying degrees of serpentinization, from the Atlantis Massif (~5 km from the LCHF) harbor microorganisms that are implicated in subsurface hydrocarbon-utilization. These microorganisms have close phylogenetic relationships to prokaryotes from hydrocarbon dominated environments and to known hydrocarbon-degraders. Additionally, we show that genes coding for hydrocarbon-oxidation and particularly for methane-oxidation are present. The presence of these genes in conjunction with our isotopic evidence indicating that abiotic methane is present, suggests that hydrocarbons of abiotic origin may support these microorganisms in deep layers of ocean crust. This new ecological niche appears to be supported by volatiles derived from the Earth's mantle.

Introduction, Results, and Discussion

The LCHF is a peridotite-hosted hydrothermal system that may driven, in part, by the exothermic serpentinization reaction (Kelley et al., 2001). The host rocks at the LCHF are serpentinized peridotite, similarly our samples from the central dome of the Atlantis Massif showed varying degrees of serpentinization of, in this case, gabbroic rocks (Blackman et al., 2006). Therefore, the mechanisms by which abiotic hydrogen and hydrocarbons are produced at the LCHF are applicable to our study site located a short distance away at central dome of the Atlantis Massif. Further, the origin of volatiles, such as methane, in gabbroic (plutonic) crust was analyzed in samples from Drillhole 735B, at the Atlantis Bank, Southwest Indian Ridge Hole (0 to 1500 meters below seafloor (mbsf)). Geochemical analysis of fluid inclusions from 735B revealed abundant abiotic methane, with some samples containing up to 40 mol % methane (Kelley, 1996; Kelley and Früh-Green, 1999). This methane was postulated to have formed by respeciation of magmatic CO_2 at ~500 to 800 °C and by serpentinization reactions at ~400 °C or greater (Kelley, 1996; Kelley and Früh-Green, 1999). The lithologies and temperatures of the central dome at the Atlantis Bank, Southwest Indian Ridge and the Atlantis Massif, Mid-Atlantic Ridge are similar; therefore, the origin and concentration of volatiles in 735B are applicable to the Atlantis Massif. These abiotic hydrocarbons could provide the carbon and energy required for prokaryotic growth.

For this study, the gabbroic central dome was sampled at Hole 1309D by drilling during the Integrated Ocean Drilling Program (IODP) Expedition 304 (0-400 mbsf) and Expedition 305 (400-1400 mbsf) (Figure 4.1, Table 4.1). We determined

Figure 4.1. Map of the Atlantis Massif.

Map of the Altantis Massif showing the locations of the Integrated Ocean Drilling Program Expeditions 304 and 305, Hole 1309D (yellow circle) and the Lost City Hydrothermal Field (green circle). Inset figure shows the location of the Atlantis Massif (yellow circle), where Hole 1309D and the Lost City Hydrothermal Field are located. Figure adapted from Blackman, D. K., J. A. Karson, D. S. Kelley, J. R. Cann, G. L. Früh-Green, J. S. Gee, S. D. Hurst, B. E. John, J. Morgan, S. L. Nooner, D. K. Ross, T. J. Schroeder and E. A. Williams (2002). "Geology of the Atlantis Massif (Mid-Atlantic Ridge, 30°N): Implications for the evolution of an ultramafic oceanic core complex." Marine Geophysical Researches 23(5): 443-469.

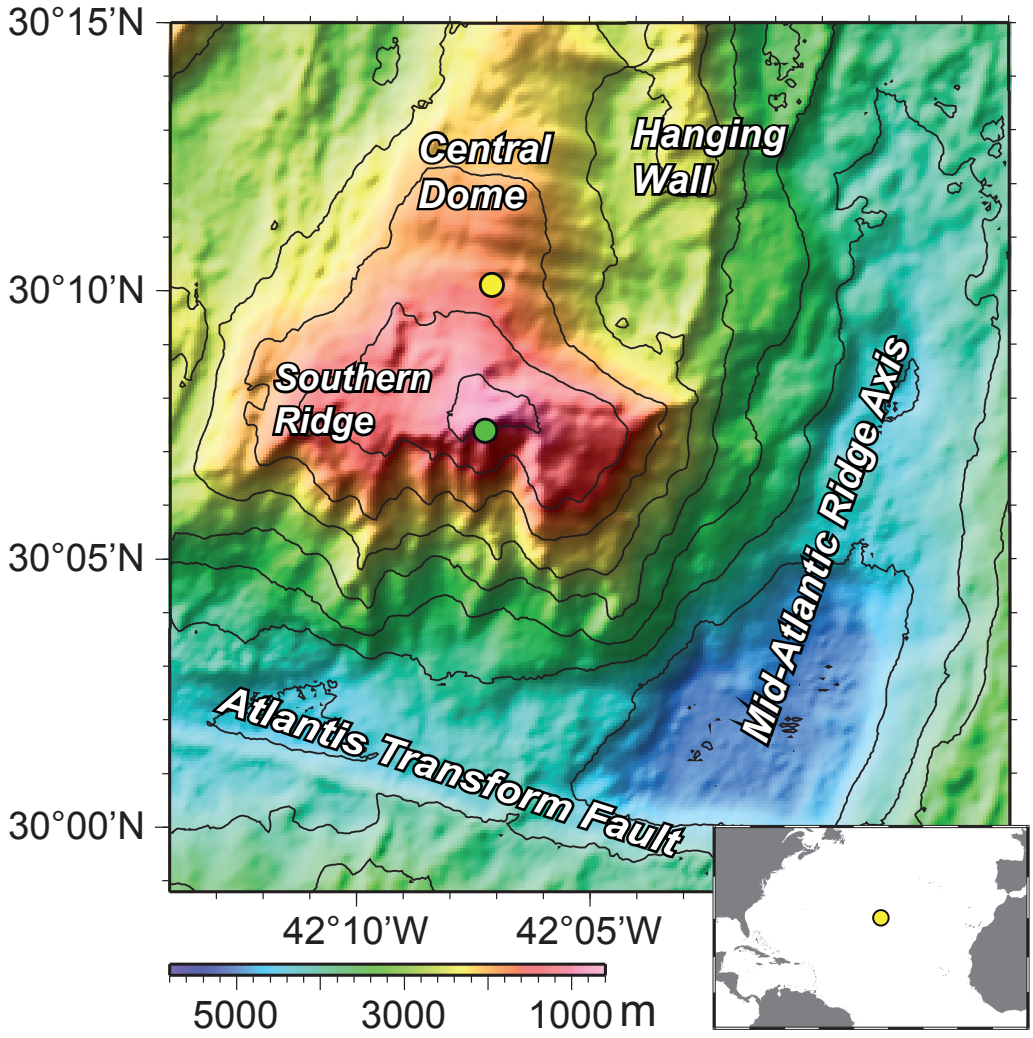
30°15'N
30°10'N
30°05'N
30°00'N
42°10'W
42°05'W
Central Dome
Hanging Wall
Southern Ridge
Mid-Atlantic Ridge Axis
Atlantis Transform Fault
5000
3000
1000 m

Figure 4.1.

Table 4.1. Samples collected for microbiological analyses from the Atlantis Massif.

Table 4.1. Samples collected for microbiological analyses from the Atlantis Massif, Hole 1309D Integrated Ocean Drilling Progam Expeditions 304 & 305.

Expedition	Site	Hole	Core sample	Section	Top (cm)	Botom (cm)	Depth (mbsf)	Rock type	Temperature (°C)	Alteration (%)	Bacterial 16S
304	1309	A	1[1]	2	45	58	0.45	Carbonate sediment	na	na	na
304	1309	D	na	na	na	na	~5[2,3]	Water sample	na	na	Y
304	1309	D	10	1	103	111	61.23	Serpentinized peridotite	14	75	Y
304	1309	D	12	2	42	50	71.69	Gabbro	14	45	Y
304	1309	D	37	2	93	101	202.83	Gabbro	17	30	Y
304	1309	D	53	1	100	111	277.4	Gabbro	21	40	Y
304	1309	D	58	1	67	73	301.07	Serpentinized peridotite	22	20	Y
304	1309	D	68	1	88	92	349.28	Gabbro	23	30	Y
304	1309	D	78	1	82	90	397.32	Gabbro	25	30	Y
305	1309	D	na	na	na	na	~397[3]	Water sample	na	na	Y
305	1309	D	80	1	18	28	401.48	Olivine gabbro	26	10	Y
305	1309	D	82[4]	1	27	39	410.47	Olivine-bearing Gabbro	26	10	na
305	1309	D	90	1	30	36	448.9	Olivine Gabbro	27	50	Y
305	1309	D	100	1	80	89	497.4	Olivine-rich Troctolite	29	10	N
305	1309	D	102	2	67	78	508.31	Troctolite	29	10	N
305	1309	D	122	2	76	89	604.24	Olivine-bearing Gabbro	33	30	Y
305	1309	D	133	3	122	132	658.73	Gabbro	36	30	Y
305	1309	D	142	3	0	13	701.05	Gabbro	38	30	Y
305	1309	D	164	1	60	74	799.6	Gabbro	43	30	Y
305	1309	D	184	1	78	85	895.78	Olivine Gabbro	48	5	Y
305	1309	D	208	4	0	12	1004.79	Gabbro	54	1	N
305	1309	D	235	2	52	63	1131.28	Olivine-rich Troctolite	63	10	N
305	1309	D	250	1	0	11	1201.5	Olivine Gabbro	68	5	Y
305	1309	D	na	na	na	na	~1215[3]	Water sample	na	na	Y
305	1309	D	273	1	116	132	1313.06	Gabbro	79	20	Y
305	1309	D	290	3	136	145	1391.01	Olivine-bearing Gabbro	102	5	N

[1]Carbonate sediment was collected solely for cell counts and was not examined any further.

[2]Units are meters above seafloor.

[3]Water samples were collected using a sterile water sampling temperature probe and served as experimental controls to determine the extent of drilling induced contamination.

[4]Sample 82 was insufficient for crushing and powdering and was not analyzed further.

Samples assayed for functional genes by microarray are highlighted.

that gabbroic samples over a 1313 meter interval harbored a low diversity of Bacteria using denaturing gradient gel electrophoresis (DGGE; Expedition 304) and terminal restriction fragment length polymorphism (T-RFLP), cloning and sequencing (Expedition 305); no Archaea were detected. Further, sequencing of DGGE bands and clones revealed a total of five different *proteobacterial* phylotypes, distinct from fluid samples (Figure 4.2). All of our bacterial clones formed clades with microorganisms from hydrocarbon-rich environments such as methane hydrates (Lanoil et al., 2001) and petroleum reservoirs (Orphan et al., 2000) (Figure 4.2).

The majority of these microorganisms were affiliated with characterized hydrocarbon- degrading *alpha, beta, and gammaproteobacterial* isolates. For example, *Ralstonia pickettii*, possesses a toluene monooxygenase (*Tbu*) allowing for growth on toluene, benzene, and alkylaromatics (Kahng et al., 2000) (Figure 4.2). AlkB genes (which oxidizes alkanes C_5-C_{12}) (Rojo, 2005) were amplified from *Pseudomonas fluorescens* when grown on hydrocarbons as substrate (1999) (Figure 4.2). Although growth of *Acinetobacter johnsonii* or *Methylobacterium aquaticum* on hydrocarbons has not been demonstrated, several *Acinetobacter* are known hydrocarbon-degraders, for example, *Acinetobacter venetianus*, utilizes C_{10} to C_{40} alkanes for growth (Throne-Holst et al., 2006) and *Methylobacterium populi* was shown to grow on methane as a sole carbon and energy source (Van Aken et al., 2004) (Figure 4.2). The remaining IODP *alphaproteobacterium* was not similar to characterized hydrocarbon-degraders, but was nearly identical (99% similarity) to an uncharacterized oil-degrading isolate (Figure 4.2). These close phylogenetic

Figure 4.2. ML phylogenetic tree of proteobacterial 16S rDNA sequences.

Maximum-likelihood phylogenetic tree of *proteobacterial* 16S rRNA gene sequences from Atlantis Massif samples. The environments from which microorganisms originated from are color coded; (see key). Branch points supported by all phylogenetic analyses (quartet puzzling support values ≥ 90%) are shown by ●; branch points supported by most analyses, but with less confidence (quartet puzzling support values 50-89 %) are shown by ○; branch points without circles are unresolved (quartet puzzling support values < 50 %). Sequences < 1200 nucleotides in length were inserted using the ARB parsimony insertion tool and are indicated by *. This proteobacterial ML tree was constructed with 1,000 puzzling steps. *Prochlorococcus marinus* (NC_009976) served as the outgroup (not shown).

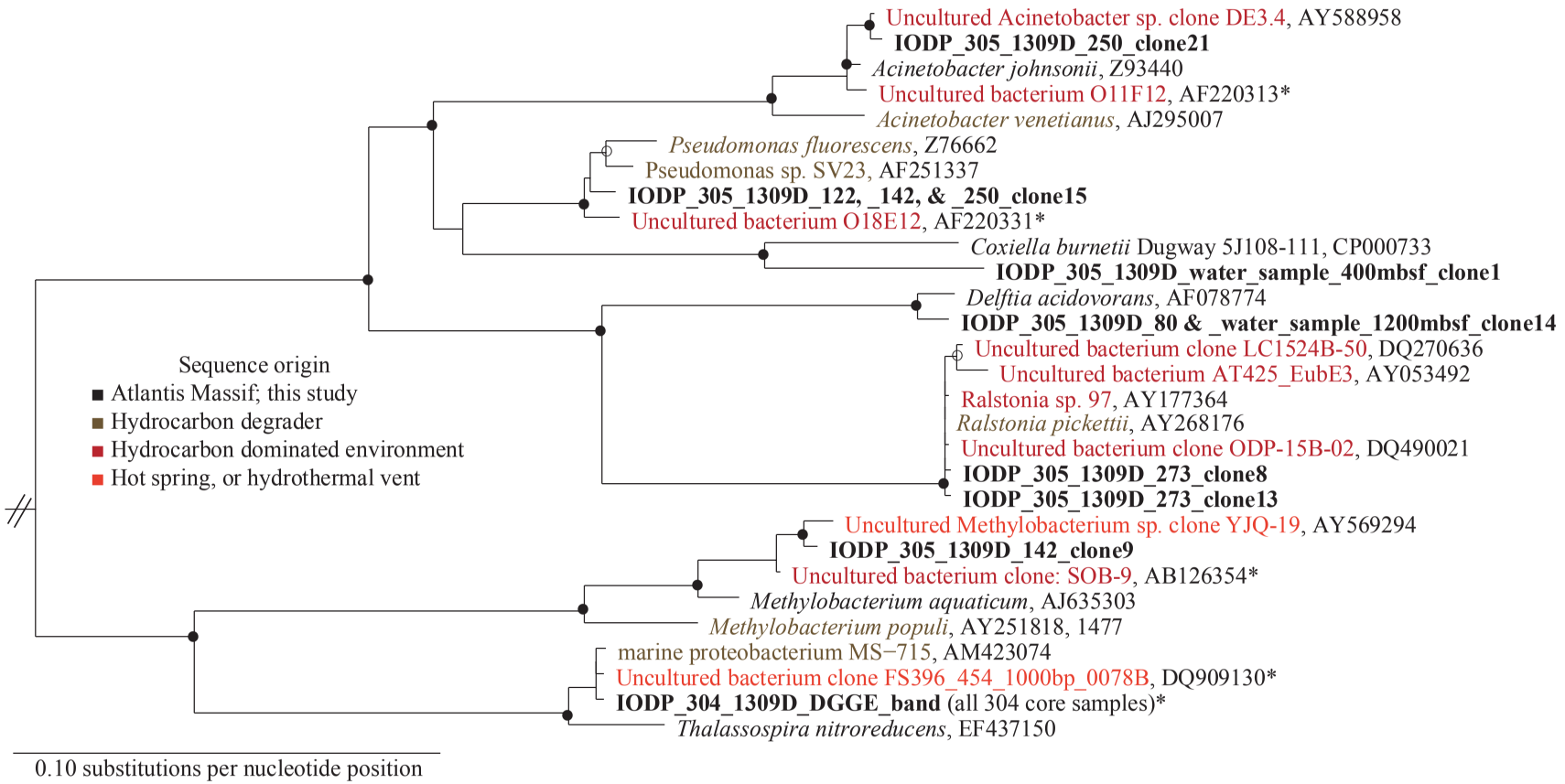
Uncultured Acinetobacter sp. clone DE3.4, AY588958
IODP_305_1309D_250_clone21
Acinetobacter johnsonii, Z93440
Uncultured bacterium O11F12, AF220313*
Acinetobacter venetianus, AJ295007
Pseudomonas fluorescens, Z76662
Pseudomonas sp. SV23, AF251337
IODP_305_1309D_122, _142, & _250_clone15
Uncultured bacterium O18E12, AF220331*
Coxiella burnetii Dugway 5J108-111, CP000733
IODP_305_1309D_water_sample_400mbsf_clone1
Delftia acidovorans, AF078774
IODP_305_1309D_80 & _water_sample_1200mbsf_clone14
Uncultured bacterium clone LC1524B-50, DQ270636
Uncultured bacterium AT425_EubE3, AY053492
Ralstonia sp. 97, AY177364
Ralstonia pickettii, AY268176
Uncultured bacterium clone ODP-15B-02, DQ490021
IODP_305_1309D_273_clone8
IODP_305_1309D_273_clone13
Uncultured Methylobacterium sp. clone YJQ-19, AY569294
IODP_305_1309D_142_clone9
Uncultured bacterium clone: SOB-9, AB126354*
Methylobacterium aquaticum, AJ635303
Methylobacterium populi, AY251818, 1477
marine proteobacterium MS−715, AM423074
Uncultured bacterium clone FS396_454_1000bp_0078B, DQ909130*
IODP_304_1309D_DGGE_band (all 304 core samples)*
Thalassospira nitroreducens, EF437150
Sequence origin
Atlantis Massif; this study
Hydrocarbon degrader
Hydrocarbon dominated environment
Hot spring, or hydrothermal vent
0.10 substitutions per nucleotide position

Figure 4.2.

relationships (Figure 4.2) suggest that microorganisms from our Atlantis Massif samples are capable of hydrocarbon-oxidation.

Analysis of functional genes using the GeoChip (He et al., 2007) microarray showed a large number of genes coding for hydrocarbon-degradation, confirming the metabolic function inferred from phylogenies (Table 4.2). We found genes coding for aerobic methane- oxidation, methane production, or anaerobic oxidation of methane by ANME Archaea (*mcrA*) (Hallam et al., 2004), and aerobic and anaerobic toluene-oxidation (Table 4.2). These findings indicate that, as predicted by phylogenies, the microflora in our plutonic crust samples have the potential to oxidize methane and toluene. Given the high methane concentrations in the analogous Hole 735B, (Kelley, 1996; Kelley and Fruh-Green, 2001) it is of particular interest that subunits of methane monooxygenases involved in aerobic methane- oxidation were detected in Hole 1309D samples.

Anaerobic degradation of hydrocarbons is suggested by the presence of genes coding for nitrate reduction. Although the majority of characterized hydrocarbon-degrading microorganisms discussed above are aerobic, both *R. picketti* (Park et al., 2002) and *P. fluorscens* (Mikesell et al., 1993) have been shown to oxidize hydrocarbons by denitrification. Further, the presence of genes coding for anaerobic toluene-oxidation implicates close relatives of *R. pickettii* in this metabolism. This is of particular interest given that IODP clones in the *R. pickettii* clade were from sample 273, from 1313.06 mbsf, a depth devoid of faults (Figure 4.3); therefore, the influx of oxygenated seawater would be minimal.

Table 4.2. Functional genes from Atlantis Massif samples.

Table 4.2. Functional genes coding for hydrocarbon production and oxidation from the Atlantis Massif, Hole 1309D.

Sample	Functional gene	Protein function	Probe source	Accession number
IODP_305_1309D_90	particulate methane monooxygenase subunit A (*pmoA*)	Predicted protein; monooxygenase activity	uncultured bacterium	BAC10328
	particulate methane monooxygenase (*pmo*)	Predicted protein; monooxygenase activity	uncultured bacterium	CAE22491
	soluble methane monooxygenase protein A gamma subunit (*mmoZ*)	Predicted protein; monooxygenase activity	Methylocystis sp. M	AAC45292
IODP_305_1309D_142	methyl coenzyme M reductase component A2 (*mcrA*)	Reduction of methyl-coenzyme M (2-(methylthio) ethanesulfonic acid) with 7-mercaptoheptanoylthreonine phosphate to methane and a heterodisulfide.	uncultured archaeon	AAQ63468
	methyl coenzyme M reductase (*mcrB*)	Reduction of methyl-coenzyme M (2-(methylthio) ethanesulfonic acid) with 7-mercaptoheptanoylthreonine phosphate to methane and a heterodisulfide	Methanobacterium thermoautotrophicum	X07794
	particulate methane monooxygenase protein A (*pmoA*)	Predicted protein; monooxygenase activity	uncultured eubacterium pAMC512	AAF08202
	soluble methane monooxygenase reductase component (*mmoC*)	Responsible for the initial oxygenation of methane to methanol in methanotrophs.	Methylomonas sp. KSPIII	BAA84756
	particulate methane monooxygenase protein A (*pmoA*)	Predicted protein; monooxygenase activity	*Methylosinus trichosporium*	AAA87220
	particulate methane monooxygenase protein A (*pmoA*)	Predicted protein; monooxygenase activity	thermophilic methanotroph HB	AAD02578
	toluene-4-monooxygenase (*T4mo*)	A four-protein complex that catalyzes the NADH- and O2-dependent hydroxylation of toluene to form p-cresol	*Pseudomonas mendocina*	AAA26001
	aryl-alcohol dehydrogenase (*areB*)	Inferred from homology; involved in the the catabolism of aryl esters, part of a toluene and xylene degradation pathway	Acinetobacter sp. ADP1	AAD34026
	chaperone	Uknown function; may be involved in anaerobic toluene metabolism	*Thauera aromatica*	CAD12889

We detected subunits of the *mcrA* operon which implies that methanogenesis could be occurring in the Atlantis Massif. However, $\delta^{13}C$ values in 735B fluid inclusions indicates that the methane is abiogenic, averaging -27 ‰ (Kelley and Fruh-Green, 2001), compared to biogenic methane at methane seeps which averages -50‰ (Orphan et al., 2001). This isotopic difference suggests that methane in plutonic crust is abiotically produced and argues against the presence of methanogens. In fact, one of the *mcrA* genes we detected was from an anaerobic methane oxidizing Archaea (ANME) (Hallam et al., 2003). Therefore, it is likely that the presence of functional genes coding for methanogenesis are in fact indicative of ANME. Further, genes coding for dissimilatory sulfate reduction (*dsrA* and *dsrB*) were present, indicating that the consortium involved in anaerobic methane-oxidation (Orphan et al., 2001) are present, and/or that hydrocarbons are degraded anaerobically by sulfate reducers (Rueter et al., 1994). The results of our phylogenetic reconstruction and functional gene survey suggest that both aerobic and anaerobic hydrocarbon-oxidation is occurring in plutonic crust.

Our data suggests that microbial methane-oxidation is occurring. To examine both the carbon source and species present we measured carbonate isotopes ($\delta^{13}C$) in carbonates over the entire 1400 mbsf interval. The $\delta^{13}C$ ranged from -2 to -7 ‰, which largely overlap the field of mantle carbon (Kelley and Früh-Green, 1999) (Figure 4.3). This indicates that the mantle is the likely source of carbon in carbonates. Further, these values indicate that methane may be present (Fruh-Green et al., 2003). The isotopic data indicates that abiotic methane, with the mantle serving as the carbon source, could supply the methane utilized in microbial methane-oxidation

Figure 4.3. Carbon isotopes from the Atlantis Massif.

Carbon isotopes from Atlantis Massif samples. Carbonate ∂13C isotopic values were measured for discrete samples from 250 to 1400 mbsf to determine the carbon species present in AM samples. Faults are indicated by dashed lines. The depth of samples collected for microbiological analyses are indicated by squares along the ordinate; unfilled squares indicate that ribosomal DNA was amplified from a sample; solid squares indicate that no ribosomal DNA was amplified. Samples that were analyzed by microarray to assay for functional genes are denoted by *.

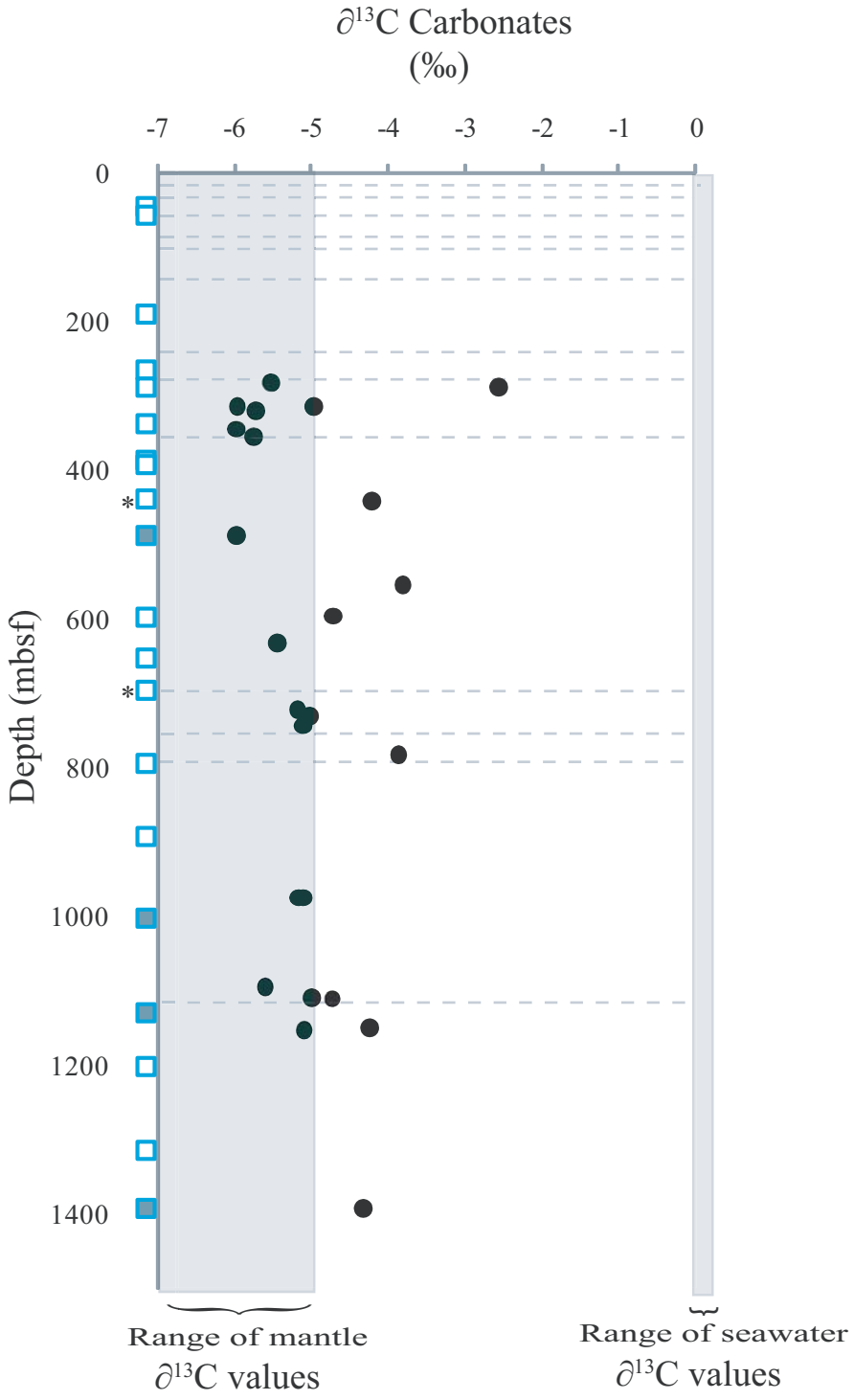
∂13C Carbonates
(‰)
-7
-6
-5
-4
-3
-2
-1
0
Depth (mbsf)
0
200
400
600
800
1000
1200
1400
*
*
Range of mantle
∂13C values
Range of seawater
∂13C values

Figure 4.3.

in subsurface gabbroic crust. Methanotrophy in particular, and perhaps methanogenic processes suggested by our data were further substantiated by a parallel study of carbon in Hole 1309D, in which alkanes > C14, such as, squalane, hopanes, steranes, pristane, and phytane were identified (Delacour et al., 2007). Squalene (diagenetically transformed to squalane (Delacour et al., 2007)) has been identified in methanotrophs (Bird et al., 1971) and hopanes are found in a variety of prokaryotes (Rohmer M et al., 1979), including methanotrophs (Neunlist and Rohmer, 1985). Steranes are ubiquitous in eukaryotes (Volkman, 2003), and although rare in prokaryotes, have been reported in a few microorganisms such as methanotrophs in the *Methylococcales* (Bird et al., 1971; Schouten et al., 2000). Pristane and phytane could originate from methanogenic Archaea (Tornabene et al., 1979). The $\delta^{13}C$ values of individual biomarkers were not determined in Atlantis Massif samples; therefore, their exact origin is not known but the results of our molecular analyses indicates that methanogens, and particularly methanotrophs are the sources of these hydrocarbons in 1309D.

Although biomarkers were present in AM samples, the dominant alkanes were unbranched, with no carbon number predominance, and showed a decrease in abundance with increasing carbon number (Delacour et al., 2007), similar to carbon compounds synthesized abiotically by Fischer-Tropsch type (FTT) reactions (McCollom and Seewald, 2006), such as at the LCHF (Proskurowski et al., 2008). Abiotic production of the unbranched alkanes (which would include methane) in AM samples is suggested. This *in situ* carbon pool could provide carbon and energy for prokaryotes in this environment.

Here we show that hydrocarbons, and particularly methane, that was likely produced by abiotic processes in the Atlantis Massif, may support a community of aerobic and anaerobic hydrocarbon-degrading prokaryotes in a novel subsurface environment. Of particular significance is the presence of methane-oxidizers. The reservoir of abiotic methane in plutonic crust and the role that prokaryotes may play in subsurface methane-oxidation have profound implications not only for Earth, but also for other planets such as Mars. Although the exact mechanism by which methane forms on Mars is not known, serpentinization reactions in the Martian subsurface have recently been proposed (Atreya et al., 2007). Therefore, similar to the Atlantis Massif, the Martian subsurface may harbor methane-consuming prokaryotes that rely on abiogenic sources of both carbon and oxidants.

Material and Methods

Sampling, cell counts, nucleic acid extraction, and whole genome amplification

Core samples were collected during the Integrated Ocean Drilling Program Expeditions 304 and 305 from 0 to 1400 mbsf. Hole 1309D temperature and percent alteration values were obtained as described by Blackman, et al. (2006). Seawater and borehole water, which served as experimental controls, were collected with a sterile water sampling temperature probe (WSTP) at five meters above the seafloor, at 397 mbsf, and at 1215 mbsf. Rock and water samples intended for molecular analyses were maintained at -80 °C until the time of analysis. To obtain cell counts from the interior of cores 304 samples were stained with 10 μg/ml 4',6-diamidino-2-phenylindole dihydrochloride (DAPI). 305 samples were fixed with 4%

paraformaldahyde for one hour during constant agitation, and stained with 1 mg/ml Acridine Orange. Samples were filtered onto a 0.2 μm filter and examined using epifluorescent microscopy (Zeiss Axiophot). For shorebased molecular analyses 304 and 305 core samples for were processed as previously described (Fisk et al., 2003). Approximately one gram of interior, powdered, rock sample was extracted using a Mo-Bio Soil DNA extraction kit according to the manufacture's protocol. Negative DNA extractions were processed in parallel to rock and water sample extractions. Genomic DNA from rocks, negative DNA extractions, and water samples from 305 were amplified using a Qiagen REPLI-g® Midi kit, following the manufacturer's protocol.

Drilling related contamination

To determine the extent of contamination from drilling fluid, 0.5 μm fluorescent microspheres were deployed in a plastic bag wedged into the core catcher that likely ruptured as the first cored material entered the core barrel. Each core collected was rinsed and assessed by microscopy (Zeiss Axiophot) to ensure that microspheres were dispersed. Contamination of the interior of the core was determined by the presence or absence of microspheres. Further water samples were obtained using a sterile water sampling temperature probe (WSTP). These samples served as molecular controls to better constrain the degree of contamination. The WSTP water collection device was initially flushed with distilled water and then sterilized with a (10%v/v) bleach solution, which remained in the tool for 0.5 h. The

tool was then rinsed with nanopure water. Approximately 5 ml of seawater was collected and immediately frozen at -80°C.

PCR, clone library construction, DGGE, T-RFLP, RFLP, and sequencing

For DGGE analysis of 304 core samples DNA fragments encoding bacterial 16S rRNA genes were amplified in TaKaRa Ex Taq (Takara Bio, Otsu, Japan) PCR cocktail (final concentration 1X) with 0.25 μM (final concentration) of the primers Eub341F with GC-clamp (5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GCC TAC GGG AGG CAG CAG-3'), and Univ907R (5'-CCG TCA ATT CMT TTR AGT TT-3') (Muyzer et al., 1993). Amplifications were carried out in a iCycler thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) with the following conditions: an initial denaturation step of 94 °C for 5 min and then 30 cycles of 94 °C for 20 s, 54 °C for 20 s, and 72 °C for 2 min. DGGE was performed with D-code systems (Bio-Rad Laboratories, Hercules, CA, USA) with 6 % (wt/v) polyacrylamide gel with denaturing gradients from 20 to 60% (100% denaturant: 7 M urea and 40% v/v formamide deionized) at 200 volts at 60°C for 4 h. DGGE of reamplified PCR products was used to check band purity. PCR amplifications were purified with QIAquick PCR purification kit (QIAGEN Valencia, CA, USA) and used as the template DNA for sequencing.

For 305 core samples bacterial 16S rDNA were amplified from rock samples and both water samples in 2X PCR Master Mix (Fermentas, Glen Burnie, MD, USA) (final concentration 1X) with 0.5 μM final concentration of 27F-B (5'-AGRGTTYGATYMTGGCTCAG-3') and 1492R (Lane, 1991). Amplifications were

carried out in a PTC-200 Thermal Cycler (MJ Research, Watertown, MA, USA) with the following conditions: 35 cycles of 94 °C for 15 s, 55 °C for 1 min, and 72 °C for 2 min, with a final extension of 72 °C for 5 min. T-RFLP reactions were carried out with the same conditions as above except the 27F-B was 5' end labeled with the phosphoramidite fluorochrome 5-carboxy-fluorescein (6-FAM). Clone library construction, screening and processing were carried out as previously described (Mason et al., 2007). Briefly, bacterial amplification products were cloned into pGEM-T Easy Vector (Promega, Madison, Wisconsin, USA) and 16S rDNA inserts were amplified with M13 primers. Full-length inserts were characterized by restriction fragment length polymorphism (RFLP) analysis. The inserts of the first 100 clones were digested with the restriction enzymes *Bsu*R1 (*Hae*III) and *Alu*I (Fermentas, Glen Burnie, MD, USA) overnight at 37 °C with the appropriate buffer and 10 units of enzyme. Few additional clones were discerned with *Bsu*R1 (*Hae*III); therefore, the remaining 200 clones were digested with *Alu*I only. Digested PCR products were resolved on a 3% agarose gel. One clone from each unique RFLP pattern was sequenced with M13F on an ABI 3730 capillary sequencer. M13R was used to generate near full-length sequences for several clones representing each phylotype. Chimeric sequences were identified with Pintail (Ashelford et al., 2005) and Mallard (Ashelford et al., 2006).

Nucleotide sequence accession numbers

Bacterial 16S rDNA sequences generated for this study have been submitted to GenBank, but have not yet been assigned accession numbers.

Phylogenetic analysis

Phylogenetic analysis was carried out as described by Mason, et al. (Mason et al., 2007). Briefly, Ribosomal RNA gene sequences from rock and water samples were searched against GenBank (Benson et al., 2005) and similar sequences were imported and aligned in ARB (Ludwig et al., 2004). Near full-length sequences, consisting of at least 1200 nucleotides, were used to construct neighbor-joining, parsimony, and maximum-likelihood phylogenetic trees. Maximum-likelihood trees of near full-length sequences were generated in ARB (Ludwig et al., 2004) using Tree-Puzzle (Schmidt et al., 2002) with the Hasegawa-Kishino-Yano model (Hasegawa et al., 1985). Shorter sequences were added to maximum-likelihood trees using the ARB parsimony insertion tool (Ludwig et al., 1998).

Functional gene analysis

Functional genes were assayed for using the GeoChip 2.0 (He et al., 2007) microarray following previously described methods (Wu et al., 2006). Briefly, amplified DNA from two core samples, IODP_305_1309D_90 and _142 was amplified in triplicate using a Templiphi 500 amplification kit (Amersham Biosciences, Piscataway, NJ) with modifications as previously described (Wu et al., 2006). Amplified DNA was fluorescently labeled with Cy5. Hybridizations were performed using a HS4800Pro Hybridization Station (TECAN, US, Durham, NC) overnight at 42 °C. Microarrays were scanned using a ProScanArray (PerkinElmer, Waltham, MA). Images were then analyzed using ImaGene 6.0 (BioDiscovery, El

Segundo, CA) to designate the identity of each spot and to determine spot quality. Data was processed as described by Wu et al (Wu et al., 2006). Protein functions were determined by searching the Universal Protein Resource (UniProt) database (Bairoch et al., 2005) .

Isotopes

Clean, handpicked calcite splits were used for isotopic analysis of carbonates. The carbon isotope composition of calcites was analyzed at GeoForschungsZentrum Potsdam in continuous flow mode with a Finnigan GasBench II (Thermo Fisher Scientific, Inc. Waltham, MA, USA) connected with a DELTAplusXL (Thermo Finnigan, Bremen, Germany) mass spectrometer. From each sample, ca 0.25 mg was loaded into 10 ml Labco Exetainer® vials. After automatically flushing with helium, the samples were reacted in phosphoric acid (100%, density 1.93) at 75° C for 60 min in a Finnigan GasBench preparation system, as previously described (Spötl and Vennemann, 2003). Carbon isotope compositions were given relative to the VPDB standard (Pee Dee Belemnite marine carbonate standard) in the conventional $\delta^{13}C$-notation, and were calibrated against three international reference standards (NBS 19, CO1, CO8). The standard deviation (1 sigma) for both standard and duplicate analyses was 0.06‰.

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Chapter 5

Dissertation Conclusion

Phylogenetic reconstruction revealed that basalt associated microorganisms are cosmopolitan in their distributions. During this analysis several ocean crust clades emerged, in particular a Marine Group I Crenarchaeota clade composed only of sequences from basalts. This suggests that basalt specialists are present in the Archaea. The remaining ocean crust clades included microorganisms from sediment and basalt indicating that numerous Bacteria are adapted to life in ocean crust.

Analysis of microbial diversity in basalts collected from Juan de Fuca, neighboring seamounts, and 9 °N, East Pacific Rise, using T-RFLP revealed that basalt microflora are distinct from seawater microflora. Further, during phylogenetic reconstruction several basalt specific ocean crust clades in the Bacteria emerged. This suggests that there are basalt specialists in the bacterial and archaeal domains.

Analysis of functional genes in basalt sample D3815F, Juan de Fuca, revealed a diversity of metabolic processes not known to be occurring in basalt. For example, genes coding for carbon fixation, methane generation and oxidation, nitrogen fixation, ammonium oxidation, denitrification, iron-reduction, and sulfate-reduction were present. The presence of carbon fixation genes and genes that code for methanogenesis are particularly significant given the oligotrophic nature of this environment. These processes may serve to underpin the trophic structure in this subsurface environment. The presence of genes coding for nitrogen fixation is compelling as basalts are composed of only a small amount of nitrogen. The presence of genes that code for denitrification suggests that basalt crust may play a role in

nitrogen removal from the marine system. Further, methane oxidation in this environment would affect the flux of methane from basalt crust to the overlying hydrosphere. The presence of genes that code for this process are significant to our understanding of carbon cycling in the deep ocean. Finally, genes that code for dissimilatory sulfate reduction and iron reduction indicate that anaerobic processes are occurring concomitant with aerobic processes. Our analysis of functional genes hints at the potential microbial metabolic diversity in marine basalts.

Beneath the basalt layer resides a substantial volume of gabbroic crust. This thesis is the first attempt to explore the species and metabolic diversity of this layer of ocean crust. Close phylogenetic relationships of gabbroic endoliths to known hydrocarbon degrading Bacteria suggested that gabbro associated microflora may oxidize hydrocarbons. The presence of functional genes that code for hydrocarbon oxidation, and in particular methane, substantiated this metabolic inference. Isotopic analysis revealed that methane, of abiotic origin, is likely present in our samples. Our findings suggest that hydrocarbons of abiotic origin may support these microorganisms in deep layers of ocean crust. This new ecological niche appears to be supported by volatiles derived from the Earth's mantle.

Analyses of prokaryotes associated with marine crust presented in this thesis provide significant insight into the distribution of basalt microflora. Further, several novel basalt ecotypes were delineated in both the Bacteria and Archaea. Functional genes present in basalt revealed the potential for a diversity of metabolic processes in this environment. Finally, the data reported here represents the entirety of what is

known about gabbroic endoliths and suggests a previously unrecognized consortium of hydrocarbon degrading prokaryotes in this layer of ocean crust.

APPENDICES

Appendix A

Media and enrichment culture preparation

Enrichment cultures were established in artificial seawater, BM_1, containing 30 g NaCl, 1 g Mg_2Cl_2•6 H_2O, 4 g Na_2SO_4, 0.7 g KCl, 0.15 g $CaCl_2$•2H_2O, 0.5 g NH_4Cl, 0.2 g $NaHCO_3$, 0.1 g KBr, 0.04 g $SrCl_2$•6H_2O, 0.025 g H_3BO_3, and 0.00072 g NaF. ASW was autoclaved and 0.344 g K_2HPO_4, 1 ml Trace Element Solution (TES) and 100 µl Vitamin Solution (VS) were added by sterile filtration to a final volume of 1 l medium. The TES consisted of 1.629 mg $NaVO_3$, 0.15 mg $K_2Cr_2O_7$, 80 mg $MnCl_2$•4 H_2O, 5 mg $CoCl_2$•6 H_2O, 20 mg $NiCl_2$•6 H_2O, 25.35 mg $CuCl_2$•2 H_2O, 60 mg $ZnCl_2$, 10.77 mg Na_2SeO_4, 150 mg RbCl, 75 mg $MoNa_2O_4$•2 H_2O, 1.26 mg $SnCl_2$, 80 mg KI, 50 mg $BaCl_2$•2 H_2O, and 15 mg Na_2WO_4•2 H_2O per liter of sterile water. The VS final concentrations per liter of BM medium are: 0.01 mg Inositol, 0.02 mg Thiamine•HCl, 0.0001 mg Vitamin B12, 0.0001 mg Biotin, 0.0002 mg Folic Acid, 0.001 mg p-Aminobenzoic Acid, 0.01 mg Niacin, 0.02 mg Ca•Pantotenate and 0.01 mg Pyridoxine. The anaerobic enrichment cultures were established in an anaerobic chamber (Coy, Grass Lake, MI) using an atmosphere of 10% H_2 / 5% CO_2 / 85% N_2. Anaerobic ASW, BM_2, media is the same as BM_1 with the following modifications: Mg_2Cl_2•6 H_2O was omitted; with 0.0071 g Na_2SO_4 and 3.35 g $NaHCO_3$ per liter. Sterile tubes containing crushed glass were equilibrated with the gas mixture for 4 to 10 days prior to inoculation. BM_2 was autoclaved, followed by pH adjustment (~ 8.0) by sparging the solution with sterile CO_2, followed by the gas mixture in the anaerobic chamber (12 h for each step). Finally 0.344 g K_2HPO_4, 1.12 g lactic acid, 1 ml TES and 100 µl VS were added by sterile filtration to a final volume of 1 l medium. BM_2 was stored in the anaerobic chamber at room temperature (~ 20°C) during the cruise. Experiments were initiated when sterilized tubes with 5 g of crushed volcanic glass were diluted with either 15 ml BM_1 or BM_2. Tubes containing 5 g crushed glass were inoculated with ~ 0.5 g fresh volcanic glass (samples D3816F and D3825). Additional tubes with and without 5 g of glass were established simultaneously by adding 15 ml of medium and served as controls.

Appendix B

Appendix B. Summary of phylogenetic affiliations of basalt sequences from bacterial OCC.

Clade	Sequence Name	Basalt sample site	Habitat of closest relative	Percent similarity	References
alpha-Proteobacteria OCC I	KBB2	Loihi Seamount	Loihi Seamount (4 sequences) and Sediments, western Pacific warm pool	99-100	(Templeton *et al*., 2005; Zeng *et al*., 2005)
	SPB-1	Loihi Seamount	Loihi Seamount (4 sequences) and Sediments, western Pacific warm pool	99-100	(Templeton *et al*., 2005; Zeng *et al*., 2005)
	SPB-2	Loihi Seamount	Loihi Seamount (4 sequences) and Sediments, western Pacific warm pool	99-100	(Templeton *et al*., 2005; Zeng *et al*., 2005)
	SPB-3	Loihi Seamount	Loihi Seamount (4 sequences) and Sediments, western Pacific warm pool	99-100	(Templeton *et al*., 2005; Zeng *et al*., 2005)
	SPB-4	Loihi Seamount	Loihi Seamount (4 sequences) and Sediments, western Pacific warm pool	99-100	(Templeton *et al*., 2005; Zeng *et al*., 2005)
alpha-Proteobacteria OCC II	JdFBGBact_66	Cobb Seamount	Hydrothermal sediments, Mid-Atlantic Ridge	98	(Lopez-Garcia *et al*., 2003)
	9NBGBact_80	9 °N	Sediments, western Pacific warm pool and Basalt, Cobb Seamount	90	(Zeng *et al.*, 2005; C.A. Di Meo-Savoie, M.M. Moeseneder, K.L. Vergin, M.R. Fisk, and S.J. Giovannoni, unpublished)
gamma-Proteobacteria OCC III	9NBGBact_73	9 °N	Sediments above a gas hydrate, Cascadia Margin, Oregon	98	(Knittel *et al*., 2003)
	9NBGBact_8	9 °N	Basalt, 9 °N	92	(C.A. Di Meo-Savoie, M.M. Moeseneder, K.L. Vergin, M.R. Fisk, and S.J. Giovannoni, unpublished)
Acidobacteria-OCC IV	9NBGBact_59	9 °N	Marine sediments	96	(Asami *et al*., 2005)
	JdFBGBact_16	Cobb Seamount	Intertidal sediments	95	(Musat *et al*., 2006)
Actinobacteria OCC V	9NBGBact_19	9 °N	Deep-sea sediments	97	(Li *et al.*, 1999)
	SM00-10D-272N	Mohns Ridge	Basalt, Southeast Indian Ridge	95	(Lysnes *et al*., 2004b)
	ODP-1155B-308	Southeast Indian Ridge	Kazan mud volcano, Eastern Mediterranean	97	(S.K. Heijs, P.W.J.J. van der Wielen, and L.J. Forney, unpublished)
	SM00-5R-466N	Mohns Ridge	Basalt, Southeast Indian Ridge	98	(Lysnes *et al*., 2004b)
Verrucomicrobia OCC VI	9NBGBact_30	9 °N	Marine sediments	91	(Freitag and Prosser, 2003)
Verrucomicrobia OCC VII	JdFBGBact_12	Cobb Seamount	Marine sediments	80, 77	(Li *et al.*, 1999)

Appendix B

References

See Chapter 2 references.

Appendix C

Appendix C. Summary of phylogenetic affiliations of basalt sequences from archaeal OCC.

Clade	Sequence Name	Basalt sample site	Habitat of closest relative	Percent similarity	References
alpha-MGI OCC VIII	HD2	HSDP, Hilo, Hawaii	Basalt, Hilo, Hawaii	99	(Fisk *et al*., 2003)
	HD74	HSDP, Hilo, Hawaii	Basalt, Hilo, Hawaii	99	(Fisk *et al*., 2003)
	JdFBGArch_3	Cobb Seamount	Basalt, Hilo, Hawaii	98	(Fisk *et al*., 2003)
	JdFBGArch_15	Cobb Seamount	Sediments, western Pacific warm pool	100	(Zeng *et al*., 2005)
	D3816, BECC1233a-3	CoAxial segment of the Juan de Fuca Ridge	Sediments, western Pacific warm pool	99	(Zeng *et al*., 2005)
alpha-MGI OCC IX	9NBGArch_26	9 °N	Basalt, 9 °N and Cobb Seamount	99	(C.A. Di Meo-Savoie, M.M. Moeseneder, K.L. Vergin, M.R. Fisk, and S.J. Giovannoni, unpublished)
	9NBGArch_36	9 °N	Basalt, 9 °N and Cobb Seamount	99	(C.A. Di Meo-Savoie, M.M. Moeseneder, K.L. Vergin, M.R. Fisk, and S.J. Giovannoni, unpublished)
	JdFBGArch_2	Cobb Seamount	Basalt, 9 °N and Cobb Seamount	99	(C.A. Di Meo-Savoie, M.M. Moeseneder, K.L. Vergin, M.R. Fisk, and S.J. Giovannoni, unpublished)
	KNIA15	Knipovich Ridge	Basalt, 9 °N and Cobb Seamount	94	(C.A. Di Meo-Savoie, M.M. Moeseneder, K.L. Vergin, M.R. Fisk, and S.J. Giovannoni, unpublished)
alpha-MGI OCC X	D3816, BECC1996b-74	CoAxial segment of the Juan de Fuca Ridge	Sediments, Pacific nodule province	99	(M.X. Xu, P. Wang, F.P. Wang, X. Xiao, unpublished)
	D3816, BECC1996b-88	CoAxial segment of the Juan de Fuca Ridge	Sediments, Pacific nodule province (2 sequences)	99	(M.X. Xu, P. Wang, F.P. Wang, X. Xiao, unpublished)
MGI-OCC XI	JdFBGArch_1	Cobb Seamount	Sediments, Aegean Sea and chimney structure, Southern Okinawa Trough	98	(M.B. Brehmer, unpublished; T. Nunoura, F. Inagaki, H. Hirayama, K. Takai, and K. Horikoshi, unpublished)
	JdFBGArch_16	Cobb Seamount	Sediments, Pacific nodule province	98	(M.X. Xu, P. Wang, F.P. Wang, X. Xiao, unpublished)
	D3816, BECC1233a-88	CoAxial segment of the Juan de Fuca Ridge	Basalt, Cobb Seamount (2 sequences), Sediments, Aegean Sea, chimney structure, Southern Okinawa Trough, and Sediments, Pacific nodule province	96-97	(M.B. Brehmer, unpublished; C.A. Di Meo-Savoie, M.M. Moeseneder, K.L. Vergin, M.R. Fisk, and S.J. Giovannoni, unpublished; T. Nunoura, F. Inagaki, H. Hirayama, K. Takai, and K. Horikoshi, unpublished; M.X. Xu, P. Wang, F.P. Wang, X. Xiao, unpublished)

D3816, BECC1196b-18	CoAxial segment of the Juan de Fuca Ridge	Nankai Trough cold-seep sediments and deep-sea mud volcanoes, Eastern Mediterranean	97-98	(S. Arakawa, T. Sato, Y. Yoshida, R. Usami, and C. Kato, unpublished; S.K. Heijs, P.W.J.J. van der Wielen, and L.J. Forney, unpublished)
D3816, BECC1196b-59	CoAxial segment of the Juan de Fuca Ridge	Basalt enrichment culture, CoAxial segment of the Juan de Fuca Ridge and deep-sea mud volcanoes, Eastern Mediterranean	99	(Fisk *et al.*, 2003; S.K. Heijs, P.W.J.J. van der Wielen, and L.J. Forney, unpublished)

Appendix C

References

See Chapter 2 references

APPENDIX D

Appendix D. Functional genes present in basalt sample D3815F determined by microarray analysis.

Genbank ID	Gene short description	Gene category	Organism
23012702	0	Carbon fixation	Magnetospirillum magnetotacticum
12407235	aclB	Carbon fixation	Chlorobium limicola
30721807	FTHFS	Carbon fixation	Methylobacterium extorquens
32307749	rbcL	Carbon fixation	uncultured bacterium
505126	rbcL	Carbon fixation	Hydrogenophilus thermoluteolus
37791353	rbcL	Carbon fixation	uncultured proteobacterium
7592878	rbcL	Carbon fixation	uncultured deep-sea autotrophic bacterium ORII-2
7592885	rbcL	Carbon fixation	uncultured deep-sea autotrophic bacterium ORII-5
37791373	rbcL	Carbon fixation	uncultured proteobacterium
21217703	rbcL	Carbon fixation	uncultured bacterium
7229162	rbcL	Carbon fixation	uncultured deep-sea autotrophic bacterium SBI-5
132036	rbcL	Carbon fixation	Rhodospirillum rubrum
7592852	rbcL	Carbon fixation	uncultured deep-sea autotrophic bacterium SBII-4
6778693	dsrA	Sulfate reduction	uncultured sulfate-reducer HMS-25
40253098	dsrA	Sulfate reduction	uncultured sulfate-reducing bacterium
FW015084A	dsrA	Sulfate reduction	lab clone
34017136	dsrA	Sulfate reduction	uncultured bacterium
34017154	dsrA	Sulfate reduction	uncultured bacterium
13898425	dsrA	Sulfate reduction	uncultured phenanthrene mineralizing bacterium
20501981	dsrA	Sulfate reduction	uncultured sulfate-reducing bacterium
FW010274A	dsrA	Sulfate reduction	lab clone
13898437	dsrA	Sulfate reduction	uncultured phenanthrene mineralizing bacterium
12667570	dsrA	Sulfate reduction	uncultured sulfate-reducing bacterium UMTRAdsr626-27
7262420	dsrA	Sulfate reduction	uncultured sulfate-reducer HMS-54
20501993	dsrA	Sulfate reduction	uncultured sulfate-reducing bacterium
34017094	dsrA	Sulfate reduction	uncultured bacterium
7262428	dsrA	Sulfate reduction	uncultured sulfate-reducer HMS-24
20502017	dsrA	Sulfate reduction	uncultured sulfate-reducing bacterium
14090290	dsrA	Sulfate reduction	Desulfomicrobium escambiense
14389123	dsrA	Sulfate reduction	uncultured sulfate-reducing bacterium
25990790	dsrA	Sulfate reduction	uncultured bacterium
40253034	dsrA	Sulfate reduction	uncultured sulfate-reducing bacterium
14389119	dsrA	Sulfate reduction	uncultured sulfate-reducing bacterium
15055587	dsrA	Sulfate reduction	Desulfococcus multivorans
22900884	dsrA	Sulfate reduction	uncultured bacterium
22999275	dsrA	Sulfate reduction	Magnetotactic cocci
14276799	dsrA	Sulfate reduction	Desulfotomaculum geothermicum
6778709	dsrA	Sulfate reduction	uncultured sulfate-reducer HMS-4
12667674	dsrA	Sulfate reduction	uncultured sulfate-reducing bacterium UMTRAdsr826-16
TPB16340A	dsrA	Sulfate reduction	lab clone
20501983	dsrA	Sulfate reduction	uncultured sulfate-reducing bacterium
FW300226A	dsrA	Sulfate reduction	lab clone

6179922	dsrB	Sulfate reduction	Solar Lake Mat Clone9065
18034323	dsrB	Sulfate reduction	Desulfacinum infernum
FW005271B	dsrB	Sulfate reduction	lab clone
13249527	dsrB	Sulfate reduction	uncultured sulfate-reducing bacterium
13561055	dsrB	Sulfate reduction	Desulfosarcina variabilis
TPB16051B	dsrB	Sulfate reduction	lab clone
15076856	dsrB	Sulfate reduction	Desulfosporosinus orientis
FW003272B	dsrB	Sulfate reduction	lab clone
14389217	dsrB	Sulfate reduction	uncultured sulfate-reducing bacterium
FW003264B	dsrB	Sulfate reduction	lab clone
28974756	dsrB	Sulfate reduction	uncultured bacterium
3892198	dsrB	Sulfate reduction	Archaeoglobus profundus
15077475	dsrB	Sulfate reduction	Desulfovibrio desulfuricans subsp. desulfuricans
FW015318B	dsrB	Sulfate reduction	lab clone
13249551	dsrB	Sulfate reduction	uncultured sulfate-reducing bacterium
FW010117B	dsrB	Sulfate reduction	lab clone
10716971	dsrB	Sulfate reduction	unidentified sulfate-reducing bacterium
TPB16055B	dsrB	Sulfate reduction	lab clone
28974734	dsrB	Sulfate reduction	uncultured bacterium
21673682	dsrB	Sulfate reduction	Chlorobium tepidum TLS
FW003269B	dsrB	Sulfate reduction	lab clone
TPB16070B	dsrB	Sulfate reduction	lab clone
13249539	dsrB	Sulfate reduction	uncultured sulfate-reducing bacterium
34017190	dsrB	Sulfate reduction	uncultured bacterium
40253070	dsrB	Sulfate reduction	uncultured sulfate-reducing bacterium
39998311	cytochrome	Metal resistance/reduction	Geobacter sulfurreducens PCA
24372200	cytochrome	Metal resistance/reduction	Shewanella oneidensis MR-1
39995722	cytochrome	Metal resistance/reduction	Geobacter sulfurreducens PCA
39996424	cytochrome	Metal resistance/reduction	Geobacter sulfurreducens PCA
24373346	cytochrome	Metal resistance/reduction	Shewanella oneidensis MR-1
39998423	cytochrome	Metal resistance/reduction	Geobacter sulfurreducens PCA
39935825	cytochrome	Metal resistance/reduction	Rhodopseudomonas palustris CGA009
2865528	cytochrome	Metal resistance/reduction	Shewanella putrefaciens
39998372	cytochrome	Metal resistance/reduction	Geobacter sulfurreducens PCA
39998004	cytochrome	Metal resistance/reduction	Geobacter sulfurreducens PCA
39997392	cytochrome	Metal resistance/reduction	Geobacter sulfurreducens PCA
23475584	cytochrome	Metal resistance/reduction	Desulfovibrio desulfuricans G20
39996164	cytochrome	Metal resistance/reduction	Geobacter sulfurreducens PCA
39937295	cytochrome	Metal resistance/reduction	Rhodopseudomonas palustris CGA009
145083	cytochrome	Metal resistance/reduction	Desulfovibrio vulgaris
15022433	mcr	Methane generation	Treponema medium
12802200	mcrA	Methane generation	uncultured archaeon 85A
38570220	mcrA	Methane generation	uncultured euryarchaeote
13259189	mcrA	Methane generation	uncultured methanogen RS-MCR04
38570178	mcrA	Methane generation	uncultured euryarchaeote
38570176	mcrA	Methane generation	uncultured euryarchaeote
799197	mcrA	Methane generation	Methanolobus oregonensis
34305116	mcrA	Methane generation	uncultured archaeon
13259303	mcrA	Methane generation	uncultured methanogen RS-ME32
7445687	mcrA	Methane generation	Methanothermobacter thermautotrophicus
34305109	mcrG	Methane generation	uncultured archaeon
20094092	mcrG	Methane generation	Methanopyrus kandleri AV19
6002398	mmo	Methane oxidation	Methylomonas sp. KSPIII
141050	mmo	Methane oxidation	Methylococcus capsulatus

6002402	mmo	Methane oxidation	Methylomonas sp. KSPIII
6002409	mmo	Methane oxidation	Methylomonas sp. KSWIII
2098700	mmo	Methane oxidation	Methylocystis sp. M
37813016	mmoA	Methane oxidation	uncultured bacterium
34915630	mmoA	Methane oxidation	uncultured methanotrophic proteobacterium
21685061	mmoA	Methane oxidation	Methylocella palustris
37813004	mmoA	Methane oxidation	uncultured bacterium
2098698	mmoA	Methane oxidation	Methylocystis sp. M
11038435	mmoA	Methane oxidation	uncultured putative methanotroph
37813020	mmoA	Methane oxidation	uncultured bacterium
7188932	pmo	Methane oxidation	Methylosinus trichosporium
7188937	pmo	Methane oxidation	Methylocystis sp. M
34733039	pmoA	Methane oxidation	uncultured bacterium
37496853	pmoA	Methane oxidation	uncultured bacterium
6424923	pmoA	Methane oxidation	uncultured eubacterium pAMC512
7578614	pmoA	Methane oxidation	uncultured bacterium FW-47
11493646	nifD	Nitrogen fixation	Azoarcus sp. BH72
29293348	nifH	Nitrogen fixation	uncultured bacterium
12001854	nifH	Nitrogen fixation	uncultured bacterium NR1611
13173333	nifH	Nitrogen fixation	uncultured bacterium
780721	nifH	Nitrogen fixation	unidentified marine eubacterium
780717	nifH	Nitrogen fixation	unidentified marine eubacterium
22449921	nifH	Nitrogen fixation	uncultured bacterium
12659182	nifH	Nitrogen fixation	Spirochaeta zuelzerae
3157500	nifH	Nitrogen fixation	unidentified nitrogen-fixing bacteria
10863131	nifH	Nitrogen fixation	marine stromatolite eubacterium HB(0898) Z02
1236929	nifH	Nitrogen fixation	Anabaena variabilis
10863141	nifH	Nitrogen fixation	marine stromatolite eubacterium HB(0697) A100
12659198	nifH	Nitrogen fixation	Treponema azotonutricium
3157624	nifH	Nitrogen fixation	unidentified nitrogen-fixing bacteria
13173301	nifH	Nitrogen fixation	uncultured bacterium
3157594	nifH	Nitrogen fixation	unidentified nitrogen-fixing bacteria
1255464	nifH	Nitrogen fixation	unidentified bacterium
22449901	nifH	Nitrogen fixation	uncultured bacterium
19070843	nifH	Nitrogen fixation	unidentified nitrogen-fixing bacteria
13173319	nifH	Nitrogen fixation	uncultured bacterium
29649383	nifH	Nitrogen fixation	uncultured nitrogen-fixing bacterium
13173335	nifH	Nitrogen fixation	uncultured bacterium
33385573	nifH	Nitrogen fixation	uncultured bacterium
780713	nifH	Nitrogen fixation	unidentified marine eubacterium
1572591	nifH	Nitrogen fixation	Desulfovibrio gigas
29293188	nifH	Nitrogen fixation	uncultured bacterium
13173305	nifH	Nitrogen fixation	uncultured bacterium
5701924	nifH	Nitrogen fixation	Paenibacillus polymyxa
3157704	nifH	Nitrogen fixation	unidentified nitrogen-fixing bacteria
12001832	nifH	Nitrogen fixation	uncultured bacterium NR1600
12001842	nifH	Nitrogen fixation	uncultured bacterium NR1605
3157506	nifH	Nitrogen fixation	unidentified nitrogen-fixing bacteria
20804123	nifH	Nitrogen fixation	Mesorhizobium loti
22450003	nifH	Nitrogen fixation	uncultured bacterium
22988609	0	Nitrification	Rhodobacter sphaeroides
7595786	amoA	Nitrification	unidentified bacterium

7544069	amoA	Nitrification	Nitrosomonas halophila
7578632	amoA	Nitrification	uncultured bacterium WC306-54
27529221	amoA/pmoA	Nitrification	uncultured bacterium
26278794	narG	Denitrification	uncultured bacterium
29652478	narG	Denitrification	uncultured bacterium
26278922	narG	Denitrification	uncultured bacterium
26278882	narG	Denitrification	uncultured bacterium
29652532	narG	Denitrification	uncultured bacterium
38427014	narG	Denitrification	uncultured bacterium
26278770	narG	Denitrification	uncultured bacterium
32308011	narG	Denitrification	uncultured bacterium
32307889	narG	Denitrification	uncultured bacterium
38427060	narG	Denitrification	uncultured bacterium
32307981	narG	Denitrification	uncultured bacterium
26278784	narG	Denitrification	uncultured bacterium
29652590	narG	Denitrification	uncultured bacterium
29652508	narG	Denitrification	uncultured bacterium
38427022	narG	Denitrification	uncultured bacterium
29652428	narG	Denitrification	uncultured bacterium
26278684	narG	Denitrification	uncultured bacterium
32307917	narG	Denitrification	uncultured bacterium
26278870	narG	Denitrification	uncultured bacterium
17385544	narG	Denitrification	uncultured bacterium
26278676	narG	Denitrification	uncultured bacterium
30269569	nasA	Denitrification	uncultured bacterium
30269577	nasA	Denitrification	uncultured bacterium
12597209	nirK	Denitrification	Alcaligenes sp. STC1
3758830	nirK	Denitrification	Hyphomicrobium zavarzinii
NBPd1-B05	nirK	Denitrification	lab clone
27125563	nirK	Denitrification	uncultured bacterium
37999212	nirK	Denitrification	uncultured bacterium
ORA-NIRK-	nirK	Denitrification	lab clone
1488172	nirK	Denitrification	Rhizobium sullae
3758901	nirK	Denitrification	Rhodobacter sphaeroides f. sp. denitrificans
28542653	nirS	Denitrification	uncultured bacterium
24421455	nirS	Denitrification	uncultured organism
24421507	nirS	Denitrification	uncultured organism
37999198	nirS	Denitrification	uncultured bacterium
24421269	nirS	Denitrification	uncultured organism
24528368	nirS	Denitrification	uncultured bacterium
28542627	nirS	Denitrification	uncultured bacterium
38455926	nirS	Denitrification	uncultured bacterium
24421271	nirS	Denitrification	uncultured organism
22252866	nirS	Denitrification	uncultured bacterium
34391466	norB	Denitrification	Nitrosomonas europaea
29466090	norB	Denitrification	uncultured bacterium
29466092	norB	Denitrification	uncultured bacterium
38373207	nosZ	Denitrification	uncultured bacterium
3057083	nosZ	Denitrification	Paracoccus pantotrophus
29125972	nosZ	Denitrification	uncultured soil bacterium
13959038	nosZ	Denitrification	Azospirillum lipoferum
4633572	nosZ	Denitrification	uncultured bacterium ProR
14994626	nosZ	Denitrification	uncultured bacterium

Appendix E

Chapter 4, Supporting Material

Cell density

Prokaryotic cell densities of interior sections of core samples over the entire 1400 m interval were below the level of detection ($<10^3$ cells cm^{-3} rock), indicating that prokaryotic cell densities are extremely low in plutonic crust. Our findings of low cell densities in Hole 1309D core samples are consistent with the densities ($1.15 \pm 0.95 \times 10^4$ cells cm^{-3}) determined in carbonate sediment sampled during Expedition 304, in nearby Hole U1309A.

Drilling related contamination

Microspheres that were dispersed during drilling operations to constrain drilling induced contamination were observed on the exterior, but not on the interior of all core samples. This suggests that drilling fluids did not contaminate samples. Sample 1309D_305_1309D_80 was the first core collected during Expedition 305. Although microspheres were not observed in the interior of any rock samples, our molecular analysis revealed that it contained microorganisms closely related to *Delftia acidovorans*. *D. acidovorans* was also found in the water sample from 1200 mbsf; therefore, we suspect that this core sample may have been contaminated by the drilling process, or from reagents (Stepanauskas, et al., 2007) and it was not analyzed further.

Reference

Stepanauskas, R. and Sieracki, M. E. Matching phylogeny and metabolism in the uncultured marine bacteria, one cell at a time. *Proc. Natl. Acad. Sci. U.S.A.* **104** (21), 9052 (2007).